IN VIVO STUDIES OF MITOCHONDRIAL tRNA MUTATIONS IN *S. CEREVISIAE*

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Abstract
Yeast mitochondria were taken as a versatile tool to investigate the pathogenecity and the mechanism of mitochondrial dysfunction due to point mutations in tRNA genes correlated in humans with neurodegenerative diseases.
The biolistic mutants LysG38A, equivalent to the G8328A mutation correlated in humans with mitochondrial encephalomyopathy, and IleT33A, equivalent to the human position 4290 of tRNAIle, were constructed and characterized. The analysis of two other yeast mutants in the anticodon region revealed that mutations involving the same position even on different tRNA genes may cause similar defects. Moreover, the efficiency of the mt-tRNA-synthetases as well as the EF-Tu to suppress the defective phenotype was tested. Finally, the importance of the nuclear background in which the mitochondrial mutation is expressed was investigated by changing the nuclear context of each mutant and quantifying the expression level of the TUF1 gene in different wild-type and/or rho° strains.
The results here described may allow the possibility to investigate the pathogenic potential of some tRNA human mutations and to search for nuclear genes that can either suppress or modify the defective phenotype.

Table of contents

Introduction

1.1 *Saccharomyces cerevisiae*

Yeasts are unicellular fungi - a large, heterogeneous group of eukaryotic organisms. The budding yeast *Saccharomyces cerevisiae*, phylum Ascomycetes, has been successfully used as an experimental system in the investigation of fundamental biological processes e.g. transcription, translation, cell cycle, and membrane transport.
Saccharomyces cerevisiae has a facultative aerobic metabolism. Thanks to its ability to ferment glucose to ethanol and carbon-dioxide, it has played a central role in food production and conservation over the centuries.
Yeasts are ideal for genetic studies of eukaryotic cell biology because they duplicate almost as rapidly as bacteria and have a genome size less than 1/100th that of a mammal. *S. cerevisiae* genome, totally sequenced in 1997, consists of 12.06 Mbp (Goffeau *et al.* 1996) of DNA and contains about 6,000 genes organized in 16 linear chromosomes. In this genome approximately 140 genes code for ribosomal RNA, 40 genes for small nuclear RNA, and 275 transfer RNA.
The average cellular diameter is 5 μm depending on the growth phase or the cell cycle, and the cell may appear with a spherical or elliptical shape. *S. cerevisiae* can proliferate in either diploid or haploid state. The diploid cells can divide by mitosis or can go through meiosis to form haploid cells. The haploid cells can divide or can conjugate (mate) with another one to form diploid cells (Fig. 1). The formation of the diploid nucleus during conjugation requires the fusion of two haploid cells followed by the fusion of their nuclei (karyogamy) activated by a particular pheromone (Rose *et al.* 1986). Yeast cells produce two different pheromones depending on the information contained in the MAT locus, *a* or α. These pheromones have a mature form which is able to bind a receptor present on the membrane surface of an opposite mating type cell.
Cell's growth rate varies according to the supply of nutrients and other factors: with normal quantities of nutrients present, diploid cells divide by mitosis (cell-cycle time is about 2 hours); under stressful conditions, diploid cells undergo meiosis producing 4 haploid cells (spores) contained in an ascus. Upon returning to favourable conditions, haploid *a* and α cells can germinate and mate to restore the diploid state.
Genetic and molecular methods such as complementation, meiotic analysis and molecular cloning can be used to characterize yeast mutants. During complementation experiments mutant strains are crossed with tester and control strains in order to reveal the mutation; the analysis of the diploid phenotype is useful to establish whether the mutation is recessive. This technique has been used to isolate endogenous as well as heterologous genes, functional analogues (orthologues), from higher eukaryotes (Leem *et al.* 2002, Noskov *et al.* 2003).
Meiotic analysis is a method used to determine if the mutation is at a single locus and to determine genetic linkage between genes. The isolation and the sporulation of the diploid is followed by tetrad analysis; because each tetrad is the product of a single meiotic event it is possible to reveal the genetic linkage from the phenotype segregation pattern.
Functional analysis of *S. cerevisiae* genome continues to provide growing information about the proteins encoded by this genome; the status of this analysis is reported in the graphic in Fig. 2.

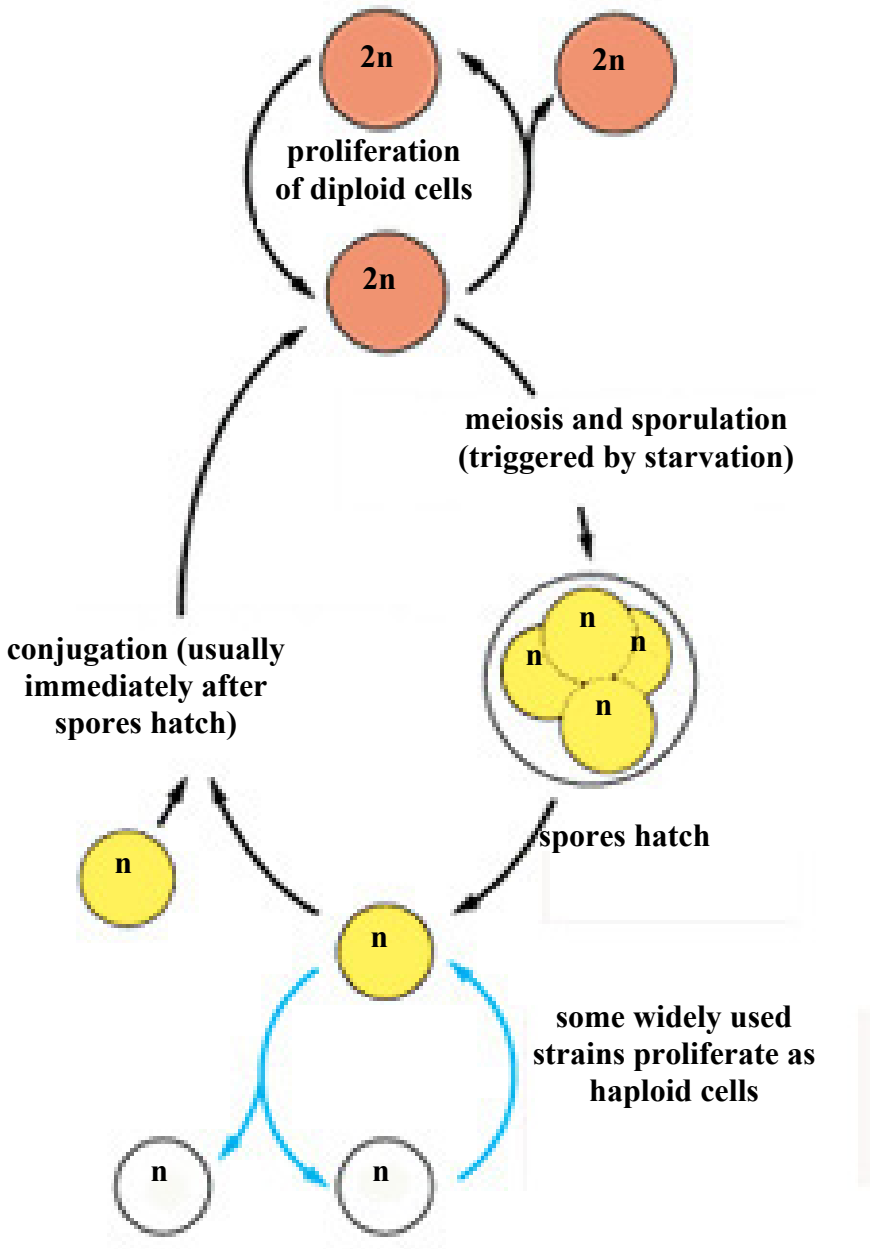
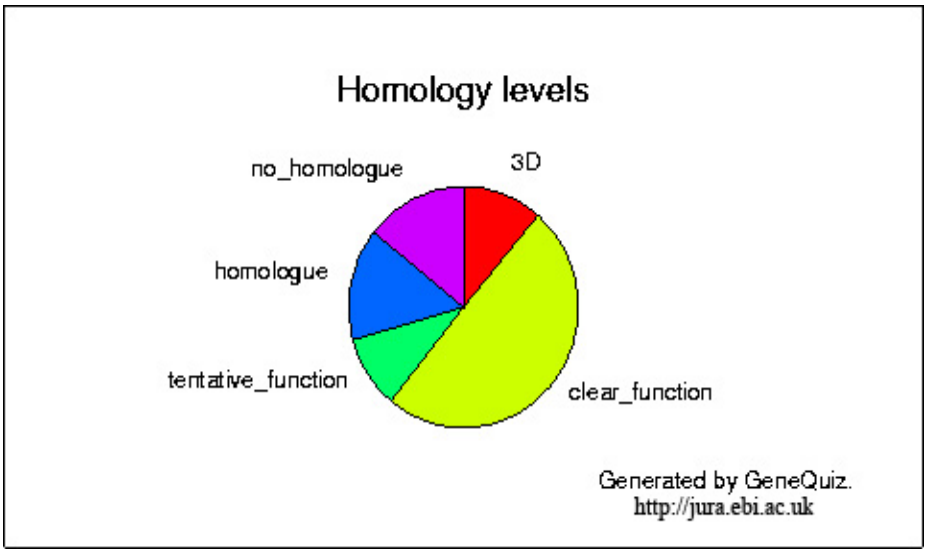


Fig. 1 *Saccharomyces cerevisiae* life cycle.



- **3D** - protein sequence has a 3D model built by homology, or a known 3D structure.
- **clear_function** - protein sequence has functional annotation derived from probable homologues.
- **tentative_function** - protein sequence has functional annotation derived from tentative homologues.
- **homologue** - protein sequence has probable homologues of unknown function.
- **no_homologue** - protein sequence has no probable homologues.

Fig. 2 Level of information known about *S. cerevisiae* genome.

S. cerevisiae has a facultative aerobic metabolism i.e. in the absence of oxygen it is able to perform the alcoholic fermentation by converting glucose into ethanol. In aerobic condition, glucose is converted to energy by the citric acid cycle and the electron-transport chain inside the mitochondria.
The carbon source present in the media can influence the metabolism of *S. cerevisiae*. In the presence of glucose, even in the presence of oxygen, this yeast has a fermentative metabolism due to the glucose repression of certain enzymes implicated in the metabolic pathway. In media containing glycerol or ethanol, only respiration is possible, which means this organism needs functional mitochondria to survive on these carbon sources.
This interesting aspect of *S. cerevisiae* metabolism permits the study of mutations which cause alterations in the mitochondrial function.

1.2 Mitochondria

1.2.1 The origin of Mitochondria

Discovered by optic microscopy observation in 1850, mitochondrion has always interested scientists because of its presence in all the eukaryotes and because the information contained in the mitochondrial genome is conserved among different organisms.

There are many theories on the origin of mitochondria. The 19th century endosymbiotic theory developed by Margulis has been extensively challenged using molecular methods. According to this theory, mitochondria originated by an ancient eubacteria endocitosis inside cellular lines destined to be eukaryotes (Margulis 1970). Phylogenetic reconstructions seemed to confirm this hypothesis even if further sequence analyses and extensive genome comparisons significantly modified it (Kurland and Andersson 2000).
Since 1998 the standard model suggests that the identified endosymbiont is Paracoccus, α-proteobacterium, and the host is an Archeon (Doolittle 1999, Gray 1992). This symbiotic relationship has been recently analysed from the biochemical point of view and seems independent from the sharing of ATP that has been produced. Therefore there are two possible models to explain this relationship: one based on an evolutionary path, initially supported by anaerobic syntrophy (Lopez-Garcia and Moreira 1999); the other, involving aerobic mutualism (Andersson and Kurland 1999), predicts that the aerobic host made pyruvate accessible to the aerobic symbiont with respiratory chain.

1.2.2 Biogenesis, structure and function of mitochondria

Mitochondria are cytoplasmic organelles present in all eukaryotic cells; they produce more than 95% of ATP through oxidative reactions and they are essential for other metabolic pathways involving mitochondrion-localized steps.
Mitochondria originated from pre-existing organelles and many factors involved in their segregation are conserved from yeast to mammals, suggesting that the mechanisms are similar. In larger eukaryotic cells segregation is performed by a cooperation between microtubules and microfilaments (Bretscher 2003). Yeast has two distinct actin nucleation systems: an Arp2/3 dependent one, for cortical patch assembly [needed for endocytosis and localized cell wall synthesis (Schott *et al.* 2002)], and a formin-dependent one for actin cables assembly, which is necessary for organelle segregation during cell division (Evangelista *et al.* 2002).
Microscopy studies suggest that mitochondrial structure can appear in two different morphological states: during inter-phase stages, mitochondria can be seen as a continuously branched reticulum (Fig. 3) of connected tube-like filaments with an approximate tube diameter of 0.5 μm (Rizzuto *et al.* 1998); during cell division they appear as ellipsoid shaped vesicles (Perkins *et al.* 1997). These changes are related to physiological conditions. The molecular bases of mitochondrial redistribution must rely on protein conformation changes that operate on relatively fast (subsecond) time scales (Knowles *et al.* 2002).

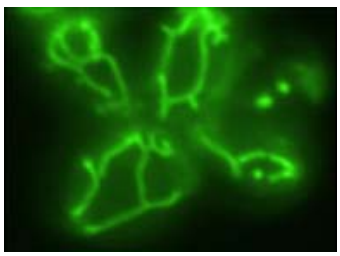


Fig. 3 Yeast mitochondria labelled with matrix localized GFP.
www.hopkinsmedicine.org/cellbio

Opposing fission and fusion events maintain the yeast mitochondrial membrane network; such processes are regulated by at least six proteins: Dnp1p, Mdv1p, Fis1p for fission, Fzo1p, Mgm1p and Ugo1p for fusion (Gorsich and Shaw 2004). Strains defective for fission or fusion show problems during meiosis and sporulation events suggesting that these mechanisms are essential to complete these processes.
Mitochondria are bounded by a double membrane. The outer membrane is permeable to small molecules and a large number of ions. The inner membrane forms cristae (Fig. 4) and contains complexes necessary for the oxidative reactions. The electron transport associated with the oxidative fosforilation is coupled with a proton gradient across the membrane which is used by the membrane ATPase complexes to produce ATP.

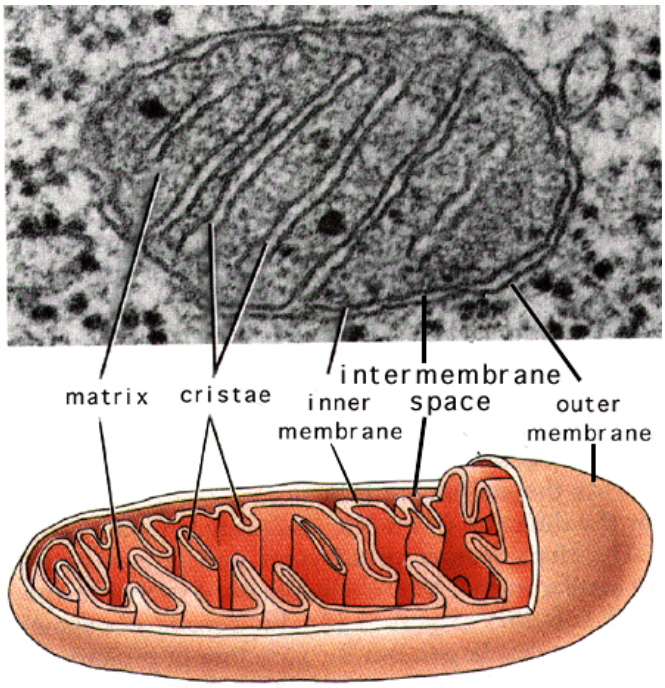


Fig. 4 Image of mitochondrial cristae. www.academicbrocklyn.cuny.edu

In the matrix of mitochondrion other processes take place, e.g. the oxidation of pyruvic acid (Kreb cycle) and fat acids.
Mitochondrial biogenesis requires the expression of about 1,000 genes, which are encoded not only by the nuclear genome but also by a mitochondrial DNA present inside the matrix in several copies.

1.3 *Saccharomyces cerevisiae* mitochondrial DNA

Saccharomyces cerevisiae mitochondrial genome is a circular molecule of DNA (Fig. 5). The molecule size may vary from 68 to 85.8 Kbp, depending on the presence of intergenic sequences and introns that differentiate one strain to another.
The *S. cerevisiae* genome contains: three genes for subunits (6, 8 and 9) of the ATPase

complexes; three genes for cytochrome *c* oxidase subunits I, II and III (COI, COII, COIII, respectively); one gene for cytochrome *b*, Cytb; and one gene for a ribosome-

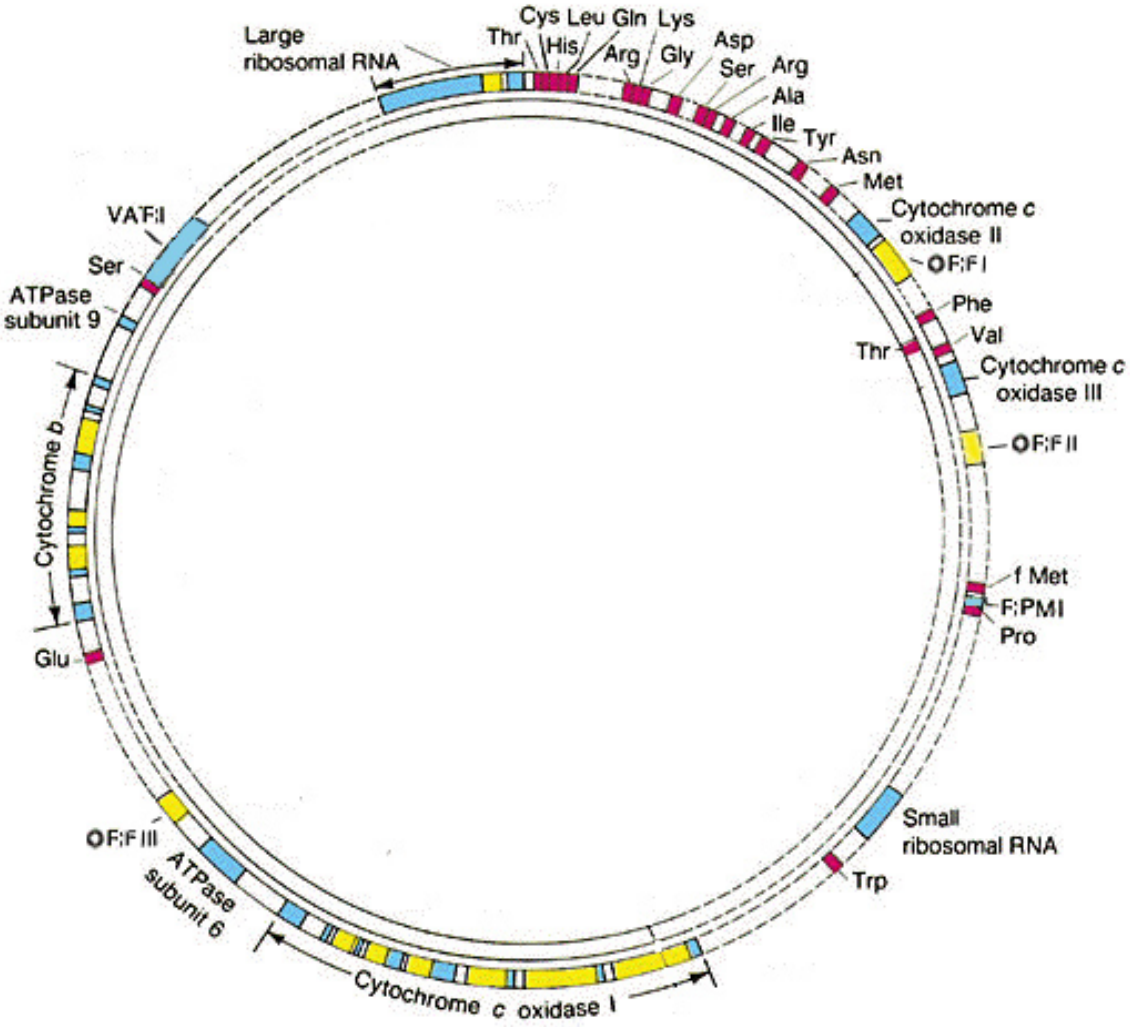


Fig. 5 Mitochondrial genome of *S. cerevisiae*.

associated protein, VAR1. The remaining regions encode for the 21S and 15S ribosomal RNAs and for 24 transfer RNAs. The 9S RNA gene (RPM1) specifies the RNA component of the ribonuclease P enzyme.

Some mitochondrial genes have introns, in particular COI, Cytb and 21S contain introns which are ORFs. Three ORFs present in the mitochondrial genome seem to have determinate functions: ORF1 and ORF2 seem to code for maturase enzymes while ORF3 code for an endonuclease subunit involved in the mitochondrial DNA recombination.

In the *S. cerevisiae* mitochondrial DNA all the genes are localized on one strand except for the $tRNA^{Thr1}$ gene. Mitochondrial DNA (mtDNA) genes are separated by long, non-coding A+T rich regions (62% of the entire genome). Almost 200 GC clusters, short stretches (less

than 25-30 bp) very rich in G+C, are interspersed in the mitochondrial genome; some of them are "hotspot" with high recombination frequency. Rearrangements of mtDNA are often due to recombination between GC clusters which form structures called "hairpin loops" (Foury *et al*. 1998). It has been observed that GC clusters may have a role also in replication (Van Dyck *et al.* 1992) and transcript maturation (Francisci *et al.* 1987).

1.4 Mitochondrial mutants

Mitochondrial mutants have a peculiar non mendelian segregation and can be of different types depending on the kind of DNA damage or on the position where the mutation is located.
When a total loss of mtDNA occurs, mutants are denominated rho°. These mutants have no functional mitochondria. Usually these mutations are associated with nuclear genes involved in the mitotic stability of mtDNA or with the presence of cell-cycle and transcription alterations (Koltovaya *et al.* 2003).
Yeast "petite" mutants have large deletions in their mtDNAs and amplify the remaining region of DNA to the wild-type genome size (Faye *et al.* 1973). These mutations denominated rho^-, occur spontaneously with a frequency of 1%, that can be increased using drugs such as Ethidium Bromide.
Strains containing point mutations or small deletions in tRNA or rRNA genes are denominated syn^-, these mutants are usually unable to perform the mitochondrial protein synthesis.
The three mutations, rho°, rho^- and syn^-, prevent the cell to breathe.
Mit^- are mutants with small deletions or substitutions in genes coding for enzymatic subunits of the respiratory chain.
Ant^R are mutants capable of growth in the presence of antibiotics or other inhibitors like tetracycline or chloramphenicol which usually interfere with mitochondrial functions.
Pet- are mutants with deletions or substitutions in nuclear genes encoding for mitochondrial proteins.

1.5 Nucleus-mitochondria interactions

In yeast, 477 proteins show evidence of mitochondrial localization; among these, 469 are nuclear encoded (Prokisch *et al.* 2004, http://ihg.gsf.de/mitop). Nuclear encoded factors include: DNA and RNA polymerases, the majority of the subunits needed for the mitochondrial inner membrane complexes, proteins implicated in the maturation of mitochondrial transcripts (Valencik and McEwen 1991), and the nuclear gene RPM2, which encodes for a subunit of the RNaseP necessary for the tRNA maturation (Kassenbrock *et al.* 1995).
Nuclear encoded proteins are synthesized on the cytoplasmic ribosomes and are imported into mitochondria. Typically, the imported proteins contain a target signal in their N-terminal presequences; these signals have the potential to form positively charged amphiphilic helices and are removed upon import (Endo *et al.* 2003). Other imported proteins contain targeting signals within the mature protein.
Mitochondrial import is mediated by membrane translocators, complexes of multiple membrane-protein subunits (which are able to function as receptors for target signals, as channels and as providers of the driving force for traslocation).
The *S. cerevisiae* general entry gate for mitochondrial precursor proteins is TOM complex (Traslocase of the Outer Membrane)(Fig. 6). This complex is formed by: receptors for precursor proteins, pore-forming protein Tom40, and small Tom proteins (Koziak *et al.* 2003). The diameter of this import channel during translocation is between 20 and 26 Å, and

this diameter may be affected by the presence of a targeting peptide (Schwartz and Matouschek 1999).
TIM23 (Traslocase of the Inner Membrane) and TIM22 are complexes present in the inner membrane (Fig. 6)(Endo *et al.* 2003). The first one mediates the translocation of presequence-containing proteins; it has an integral membrane protein Tim23 that forms a dimer depending on the potential across the inner membrane, and dissociates at the presence of presequences. TIM22 is a complex, forming a pore of 16 Å, needed for the insertion of presequence-less

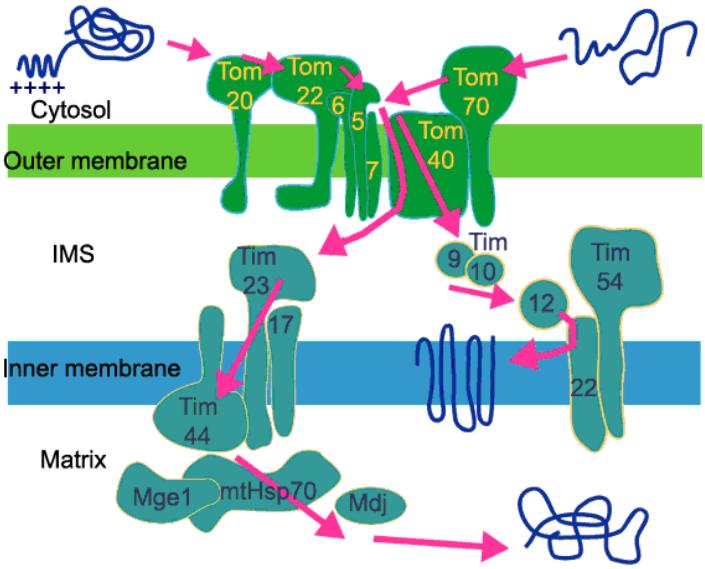


Fig. 6 Mechanism of mitochondrial import of nuclear encoded proteins. Tom20 is the general receptor for proteins containing presequences, while Tom70 recognizes targeting signals present in the mature protein and/or those in the presequences.
www.cgm.cnrs-gif.fr

polytopic membrane proteins like ADP-ATP carriers and some Tim proteins. Import across the inner membrane requires membrane potential $\Delta\Psi$ and/or ATP.Recent studies described a new mechanism of mitochondria to nucleus stress signaling initiated by mtDNA or membrane damage and disruption of $\Delta\Psi_m$ (Biswas *et al.* 2003). This cellular modulation is called retrograde regulation, which is an homeostatic or stress response mechanism to adjust metabolic and biosynthetic activities to the alterations in the mitochondrial state (Sekito *et al.* 2000).

1.6 Mitochondrial DNA: replication and transcription

S. cerevisiae cells contain 30-100 copies of mtDNA, which replicates in a rolling circle mode starting from at least seven separate origins (rep and ori) containing highly conserved sequences (Wallace 1982). Concatenamers are the obligatory intermediates for partitioning of mtDNA during cell division, these concatenamers are processed into circular monomers upon transmission from mother cells to buds. One hypothesis suggests that in yeast there is a selective transmission of a few clones (physically connected) of mtDNA; this means that the alleles of the mitochondrial genome quickly segregate during mitosis and within 10-20 cell cycles mtDNA becomes genetically homogeneous (homoplasmic) (Ling and Shibata 2004).

MtDNA maintenance seems to be correlated with the mitochondrial histone Abf2p which appears to play an essential role in recombination and copy number determination (Cho *et al.* 2001).
This genome is transcribed by the mitochondrial RNA polymerase (mtRNAP). In *S. cerevisiae* this enzyme is composed of two nuclear encoded proteins, the core Rpo41 and the mitochondrial transcription factor Mtf1 (Matsunaga *et al.* 2004). The transcription is started from an highly conserved nonanucleotide (5'-TATAAGTAA [+2]) promoter sequence. It has been found that this sequence plus more than 9 nt of nonconserved sequence 3' to the promoter are essential for mitochondrial specific transcription (Biswas 1999). Transcription regulation seems correlated with conformational changes involving a region of approximately 300 nt 5' to the promoter.
Microarrays and other experiments indicate that in yeast the main determinant for gene regulation is the transcription rate; the key factor seems to be the modulation of mRNA stability (Garcia-Martinez *et al.* 2004). Usually transcripts are maturated by cleavage at the 5'-end and at the 3'-end; 3' cleavage endonucleases recognize a specific sequence (5'-AAUAAUAUUCUU-3') and this cleavage seems to protect mRNA from degradation by esonucleases.
Some mitochondrial genes require intron-specific splicing factors, which are nuclear encoded or derivate from some mitochondrial intron sequences. An example of mitochondrial splicing is the excision of introns contained in the cytochrome b and subunit I of cytochrome oxidase made by the leucyl-tRNA synthetase (NAM2p)(Houman *et al.* 2000).
Moreover, the three-dimensional structure of GC-clusters is a maturation signal for some pre-tRNAs (Francisci *et al.* 1987).

1.6.1 Mitochondrial tRNAs

The tRNAs are essential molecules for the protein biosynthesis in all living organisms.
They have specific structural properties required to interact with various proteins like the aminoacyl-tRNA-synthetases, the translational initiation and elongation factors, and the ribosomal machinery (Helm *et al.* 2000). Their sequences have conserved and semi-conserved information which are essential for interactions and for folding into the "canonical" cloverleaf secondary structure (Fig. 7A) and further into a three-dimensional L-shaped structure (Fig. 7B). The "canonical" secondary structure is formed by base-pairing regions denominated stems and single strand regions denominated loops. A canonical tRNA contains: the acceptor stem, required to bind the aminoacid; the D branch, in which the loop contains a diidrouridine; the anticodon branch; the T branch, in which the loop contains the pseudouridine (Ψ); and a variable loop.
The L-shape is due to tertiary interactions and is essentially formed by an anticodon branch (consisting of the D-stem and the anticodon-stem) and by an acceptor branch (consisting of the acceptor stem and the T-stem).
The majority of the yeast mitochondrial tRNA genes are localized in a region of 20 Kbp between 21S and OXI1 genes. This region contains four promoters, consequently, tRNA genes are transcribed as polycistronic pre-tRNAs that require maturation at the 5' and at the 3'-end. While the RNase P, a ribonucleoprotein enzyme, removes the 5'-end sequence, the La protein binds the 3' trailer sequence and protects it from exonucleases. The maturation at the 3' terminus is still the subject of studies; apparently this processing is due to an endonuclease, the RNase Z (Marchfelder *et al.* 2002).
tRNAs are substrates of some modifying enzymes. Modified bases vary from 7 to 15 among tRNAs, some are tRNA specific while others are conserved, like the diidrouridine in the D-loop. Some of the modifying enzymes have been identified; for example, Mod5 is a protein that modifies the D-loop uridine in diidrouridine (Xing *et al.* 2002), and N^2,N^2-

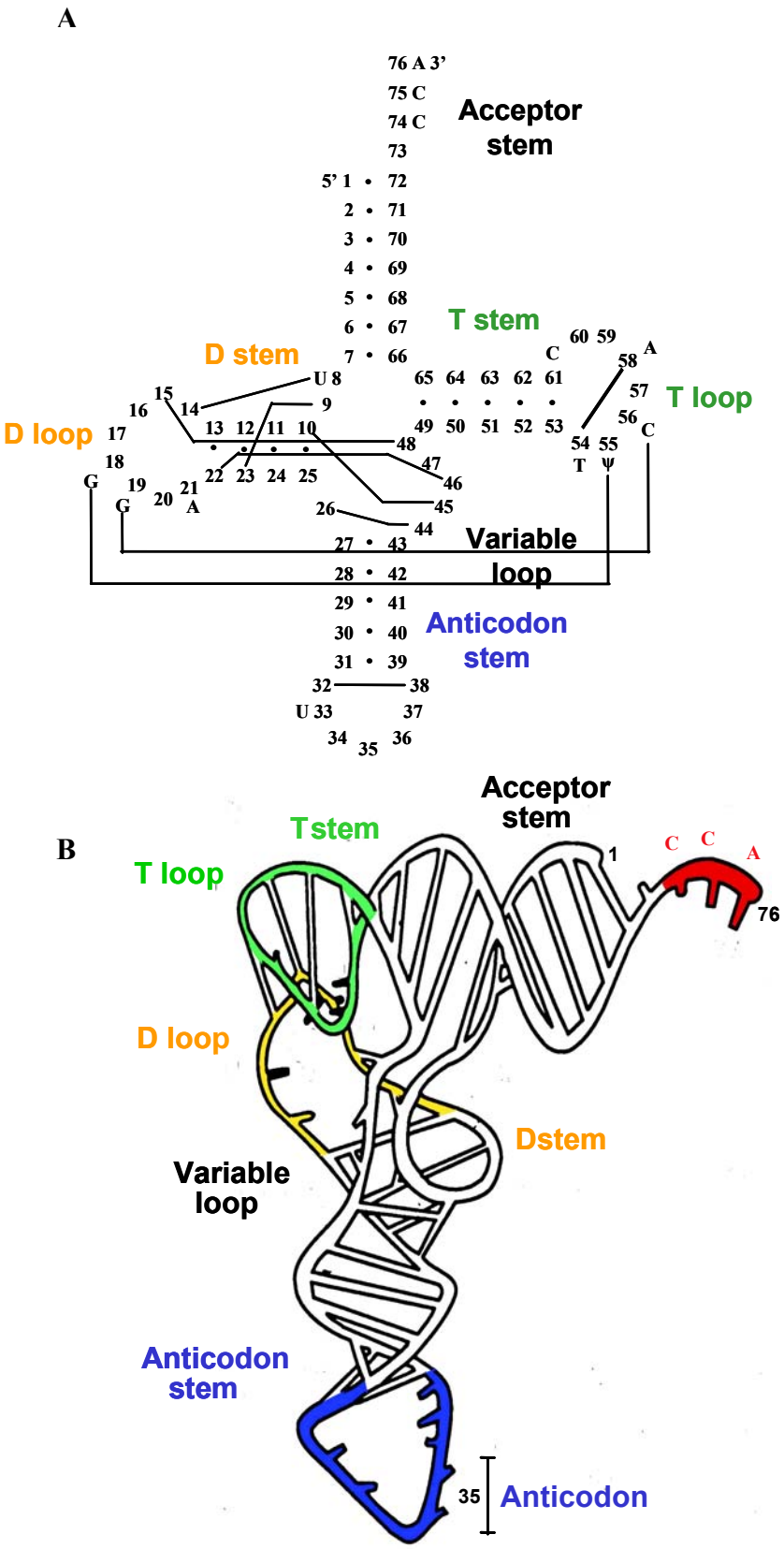


Fig. 7 Secondary (A) and tertiary (B) structure of tRNA.

dimethylguanine tRNA methyltransferase is able to catalyse the modification of G26 in many tRNAs.
Mitochondrial tRNAs have less modifications compared to their cytosolic counterparts; this suggests that this modifications are of higher importance as they are retained by evolution (Helm *et al.* 1999). Moreover, tRNAs modifications are known to stabilize tRNA structure by blocking or adding hydrogen bonds.
The ATP(CTP):tRNA nucleotidyltransferase adds the CCA to the 3' termini of all the mitochondrial tRNAs (Wolfe *et al.* 1999).
tRNAs are aminoacylated at their 3' ends by specific aminoacyl-tRNA synthetases; these enzymes have been divided in two classes depending on the catalytic fold, identifiable peptide sequence motifs, and distinctive mechanistic features (Francklyn *et al.* 2002). Aminoacylation is a two step reaction in which the amino acid condensates with ATP to form an activated amioacyl adenylate intermediate, then the amino acid is transferred to the 3'-terminal ribose of the tRNA. The interactions between the synthetases and the cognate tRNA is based on the presence of identity elements within the tRNA molecule. In general these elements are contained within the acceptor stem and the anticodon region (Stello *et al.* 1999)
The mitochondrial genome encodes for all the tRNAs necessary for the organelle protein synthesis. According to the wobble hypothesis the number of required tRNAs is 32 but only 24 tRNA genes are encoded by the mtDNA; subsequently, mitochondria have a different codon usage (Wallace *et al.* 1982). It has been demonstrated that each tRNA can read two or four codons; this capability is correlated with the presence of an uridine in the 5' position of the tRNA anticodon. There are two possible hypotheses to explain this mechanism: the uridine in the 5' position could recognize all the possible four bases or just the first two positions of the anticodon are recognized (Martin *et. al.* 1990).

1.7 Mitochondrial protein synthesis

Translation within the mitochondrial matrix depends upon mRNA specific translational activators recognizing targets in the mRNA 5'-unstranslated leaders and mediating mRNA interactions with mitochondrial ribosomes (Naithani *et al.* 2003).
Mitochondrial ribosomes derived from proteins encoded by nuclear genes (the exception is Var1p an essential protein for ribosome biogenesis) and ribosomal RNAs (21S and 16S) transcribed from mtDNA. Other proteins are probably involved in the ribosome biogenesis: Mtg1p, for example, seems to catalyse a modification of a ribosomal protein that interacts with domain V of 21S rRNA or transiently stabilizes an RNA fold (Barrientos *et al.* 2003).
Translation consists of three steps: 1) binding of aminoacyl-tRNA, 2) peptide bond formation, and 3) translocation of the bound tRNAs and mRNA relative to the ribosome. The proofreading and selection of the cognate tRNA at its mRNA depend on codon-anticodon interaction and the identification of structural elements of tRNA by the ribosome (Schnitzer and Ahsen 1997).
The initiation involves a set of proteins denominated eukaryotic initiation factors (eIFs)(Abbot and Proud 2003). This process starts with the positioning of the ribosomal P-site at the start codon, helped by some eIFs factors, and then the initiator methionyl-tRNA recognizes the start codon.
During the elongation the amino acids are added to the growing polypeptide chain. eEF1A (EF-Tu) is responsible for delivering the aminoacyl-tRNA to the ribosomal A-site. This activity is dependent on GTP and eEF1B (EF-Ts) is the factor promoting the regeneration of the eEF1A-GTP. eEF1A forms a ternary complex with tRNA and GTP. In the general model of the ternary complex (well conserved in all organisms), the tRNA T-stem contacts the domain III of the factor and the acceptor stem contacts the switch regions of domain I. The A-

base of the CCA-end binds in a pocket on the surface of domain II and the aminoacyl-ester bond is located between domains I and II (Andersen *et al.* 2003).
The termination occurs when a stop codon is positioned in the A-site. The eRF complex promotes the cleavage of the bond between the peptide and the tRNA, releasing the newly synthesized peptide.
The high fidelity and the speed of the protein synthesis *in vivo* is achieved through the participation of GTP-dependent elongation factors. Over expression of eEF1A in yeast leads to decreased translational fidelity (Song *et al.* 1989). It has been found that eEF1A has numerous other functions, e.g. the cytoskeletal remodelling. Moreover, it is an essential protein in yeast in which null mutations of this protein are lethal (Cottrelle *et al.* 1985).

1.8 Human mitochondrial DNA

The human mitochondrial DNA is a circular molecule of 16.569 Kbp (Fig. 8).

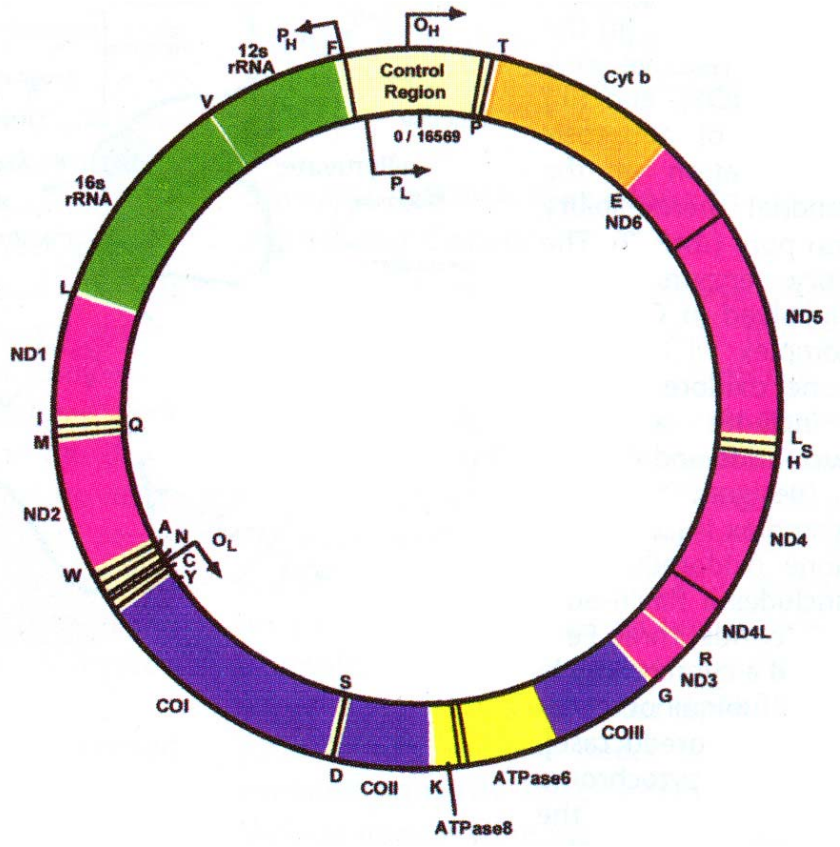


Fig. 8 Human mitochondrial genome (www.mitomap.org). Genes encoding for different subunits of the same oxidative phosphorylation complex are indicated by the same colour: ND1-2-3-4-4L-5-6 for complex I (NADH: ubiquinone oxidoreductase) are in pink; cytb for complex III; COI, II, and III for complex IV are in blue; and ATP6 and 8 for complex V are in yellow. The tRNAs are indicated by their cognate amino acid single letter code. rRNAs are indicated in green. The H- and L- strand origins (O_H and O_L) and promoters (P_H and P_L) are shown.

Each cell contains hundreds of mitochondria and thousands of mtDNA molecules. As in yeast, the human mtDNA encodes only for some subunits of the mitochondrial oxidative phosphorylation complexes, for the small and large rRNAs and for 22 tRNAs. There is also a non coding region denominated D-loop.
Human mtDNA is a double strand molecule; the two strands can be separated by a density gradient and on the basis of their nucleotide composition are termed heavy (H) and light (L) strand (or leading- and lagging- respectively). The H-strand encodes for most of the genes.
Recent studies demonstrated that the mtDNA in mammals replicates by a strand coupled (θ) mechanism arising from multiple origins (Bowmaker *et al.* 2003). This mechanism implies a bi-directional initiation zone downstream a powerful replication fork barrier. O_H (previously designated as an origin for the replication) and D-loop may act as a terminus for the replication by forming a structural barrier.
Sequences without coding functions are absent in animal mtDNA. Transcription probably starts at single site for the H- and L-strand and continues symmetrically around the genome. Moreover, it has been proposed that tRNA genes, interspersed among the ORFs, can be used as recognition sites for processing of the polycistronic transcripts (the punctuation model). rRNAs are modified post-transcriptionally by the addition of short adenyl stretches and mRNAs are polyadenylated by the mitochondrial poly (A) polymerase (Smeitink *et al.* 2001).

1.9 Mitochondrial alterations and pathology

The mitochondrial genome is very sensitive to mutations; the mutation rate of mtDNA is higher than that of nuclear DNA (Laderman *et al.* 1996). During the oxidative phosphorylation system (OXPHOS) reactions about 1 to 4 % of oxygen uptake is converted to oxygen radicals. It has been observed that mtDNA accumulates 16 times more oxidative damage than nuclear DNA (Wallace 1992).
The OXPHOS paradigm is a series of concepts providing an hypothesis to explain degenerative diseases. The four basic concepts in the OXPHOS paradigm are: OXPHOS produces mitochondrial energy, OXPHOS is the main source of energy for different tissues, OXPHOS genetic involves nuclear and mitochondrial genes, OXPHOS declines with age (Wallace 1992).
Mutations in the mtDNA can also cause alterations in the cytoplasmic Ca^{2+} metabolism in two ways: indirectly by reducing the levels of ATP, which is also used by the Ca^{2+}-dependent ATPases to pump Ca^{2+} into intracellular stores or out of the cell; and directly by perturbing the mitochondrial membrane potential and therefore the Ca^{2+} uptake (James and Murphy 2002). The cytoplasmic Ca^{2+} concentration (normally around 0.1-1 μM) is also an important signal for a number of pathways including hormone regulation.
Among the metabolic diseases, mitochondrial disorders are the most frequent with an estimated incidence of 1 in 10,000 live births (Smeitink *et al.* 2001). A variety of degenerative disorders may be associated with defects in OXPHOS; these defects include base substitutions in tRNA and protein coding genes, insertions or deletions in mtDNA, and nuclear mutations that cause alteration in the mitochondrial protein synthesis or mtDNA large deletions. It is estimated that mtDNA mutations are responsible for 20 % of OXPHOS deficient patients, the remaining portion must harbour nuclear gene mutations (Smeitink et al. 2001). On the www.mitomap.org web site there is an up-to-date database reporting mtDNA mutations correlated with pathologies.
One example of clinical phenotypes associated with mitochondrial mutations is the Leber ereditary optic neuropathy (LHON). 19 mutations have been associated with this neuropathy, 5 appear to play a primary role in causing disease. These mutations are transitions in ND6, ND4 and cytb genes (Wallace 1995). Other clinical phenotypes associated with mitochondrial mutations are: Kearns-Sayre syndrome (KSS), chronic progressive ophthalmoplegia (CPEO),

neurogenic muscle weakness ataxia and retinitis pigmentosa (NARP), mitochondrial encephalopathy lactic acidosis and stroke like episodes (MELAS), myoclonus epilepsy with ragged red fibres (MERRF), diabetes mellitus, Leigh syndrome, Huntington's disease, and even Alzheimer's and Parkinson's diseases (Wallace 1992).
As reported in table 1, nowadays more than 90 different mutations in tRNA genes have been correlated with various neuromuscular and neurodegenerative disorders. Although tRNA genes represent 10 % of mtDNA, their mutations account for 75 % of mtDNA-related diseases. The most frequently observed mutations are A3243G in the tRNALeu(UUR) gene, and A8344G in the tRNALys gene, both associated with MELAS syndrome.
Mitochondrial pathologies are recognized for their typical maternal inheritance, in fact less than 0.1 % are contributed by the sperm (Wallace 1992).
Each cell contains hundreds of mitochondria and thousands of mtDNA molecules; therefore, cells can contain a mixture of mutant and normal mtDNA (heteroplasmy).

Table 1 Mutations in mitochondrial tRNA genes correlated with human pathologies (www.mitomap.org, 2004).

tRNA	Number of mutations	TRNA	Number of mutations
tRNA Ala	1	tRNA Lys	12
tRNA Asn	3	tRNA Met	2
tRNA Cys	1	tRNA Phe	4
tRNA Gln	3	tRNA Pro	1
tRNA Glu	1	tRNA Ser (AGY)	2
tRNA Gly	5	tRNA Ser (UCN)	8
tRNA His	3	tRNA Thr	4
tRNA Ile	12	tRNA Trp	5
tRNA Leu (CUN)	4	tRNA Tyr	2
tRNA Leu (UUR)	21	tRNA Val	4

The mutant and normal mtDNAs are randomly segregated into the daughter cells during cell divisions. Over many cell divisions the proportion of mutant and normal mtDNAs can drift to either pure mutant or pure normal (homoplasmy) (Fig. 9).
As the severity of the pathology is related to the proportion of mutated mtDNA molecules and to the energetic threshold of various tissues and organs, the phenotypic effect of mutation varies. OXPHOS capacity declines with age; consequently, individuals born with OXPHOS mutations have the tendency to reach the energetic limit of an organ and to present disease symptoms earlier.

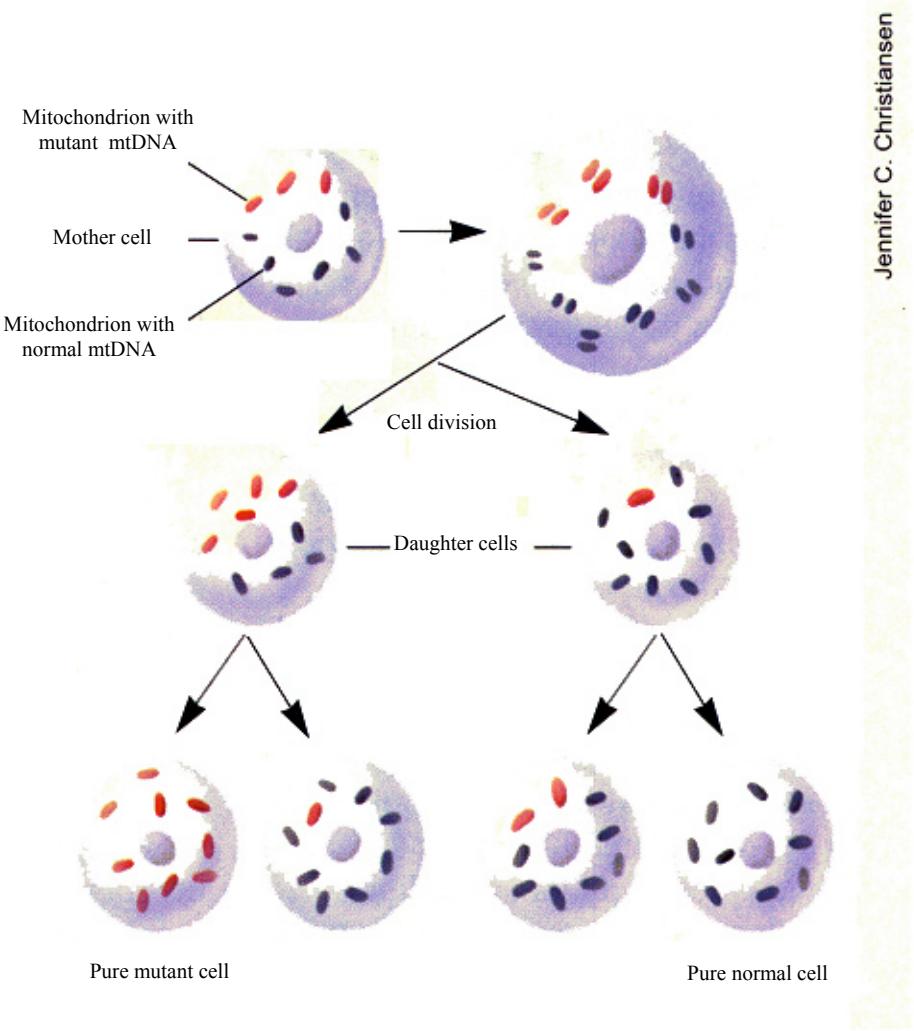


Fig. 9 During the cellular division process, mitochondria are randomly distributed into daughter cells; consequently, their mtDNA can become genetically homogeneous.

1.10 New tools and models to study mitochondrial diseases

Difficulties in the study of mitochondrial pathologies are related to: the different phenotype of patients, often due to the genetic background of the mutations (Jacobs and Holt 2000); the difficulty in identifying the genetic origin of the dysfunction that may also come from

mutations in nuclear genes; and the proportion of mutated molecules, since structural alterations can occur in a small fraction of mtDNA population (Laderman *et al.* 1996).
To study these pathologies several laboratories developed human cybrids, in which an enucleated cell is fused with cytoplasts from a rho° cell. Different cultures of cybrids with various proportions of mutated mtDNA molecules are useful to investigate which amount of normal molecules are required for the mitochondrial function.
Cybrids are often used to test the effect of the expression of transfected nuclear genes. In some cases it is possible to transfect heterologous genes conserved during the evolution. An application of this technique is the analysis of the expression of the algal ATPase 6 gene from the *Chlamydomonas reinhardtii*, in which it is nuclear encoded, in human cybrids. These cells have a pathogenic mutation in the mtDNA-encoded ATPase 6 gene (Ojaimi *et al.* 2002). The tranfected cybrids gained only a minimal increase of cell growth rate due to the moderate complementation of function by the algal protein. Endogenous proteins could be engineered in order to be expressed in human cells but numerous obstacles need to be overcome to adapt this approach to gene therapy.
Model organisms, such as mice, helped to determine the pathophysiology of mitochondrial diseases. Recent studies provide strong evidence that both reduced mitochondrial energy output and increased mitochondrial oxidative stress are important factors in the physiopathology of mitochondrial diseases (Esposito *et al.* 1999). Using cre-loxP recombination system to disrupt nuclear genes for a transcription factor, Wredenberg *et al.* developed several mouse models for mtDNA disorders. These mice are used to reproduce important pathophysiological features found in human patients with mitochondrial myopathy in order to finally correlate the mitochondrial mutations to clinical syndromes (Wredenberg *et al.* 2002).
Gene therapy seems to be almost the only way to cure patients with genetic disorders. More than 3,500 patients have been treated over the last 20 years throughout U.S. and Europe (Mountain 2000), although there is still no evidence of its efficacy. Gene therapy is going to become a reality within the future, taking advantage of the recent improvements in "genomics" and "proteomics" (Alesci *et al.* 2002).

1.10.1 *S. cerevisiae* as model to study mitochondrial mutations

S. cerevisiae has been successfully used to analyse mitochondrial mutations. Some of its advantages are:

1. the facultative aerobic metabolism;
2. an homoplasmic content of mtDNA molecules;
3. a rapid cell cycle;
4. a well characterized genetic and molecular biology;
5. the possibility to introduce mitochondrial mutations.

Fox *et al.* in 1991 reported a technique to transform yeast mitochondria, denominated Biolistic Transformation. This technique uses a device for micro projectiles which is necessary to shoot tungsten particles inside yeast cells. These projectiles are prepared with exogenous DNA to transform yeast mitochondria. The homologous recombination, which is frequent in *S. cerevisiae*, is one of the main advantage of using this model.
The biolistic transformation has been successfully used to analyse the effect of the introduction in the yeast mitochondrial tRNALeu of three point mutations (Feuermann *et al.* 2003). As the human tRNALeu (UUR) sequence (Fig. 10A) and structure are very similar to the *S. cerevisiae* ones, mutations A14G, C26T and T69G, equivalent to the human pathogenic A3243G, C3256T and T3291G respectively, have been characterized (Fig. 10A).

The defective phenotype of these mutants was strong, in fact these mutants were not able to grow on glycerol containing media (respiratory media). At position 69 also the non-

pathogenic substitutions were introduced and these mutants showed no growth defect on respiratory media. This result suggested a good correlation between the pathological mutations in humans and the defective phenotypes in yeast.
In the tRNALeu mutants it has been analysed the suppression by the EF-Tu protein. TUF1 gene cloned into a multi-copy vector rescued the defective phenotype of LeuA14G, C26T and T69G mutants (as an example the C26T mutant suppression is shown in Fig. 10C).

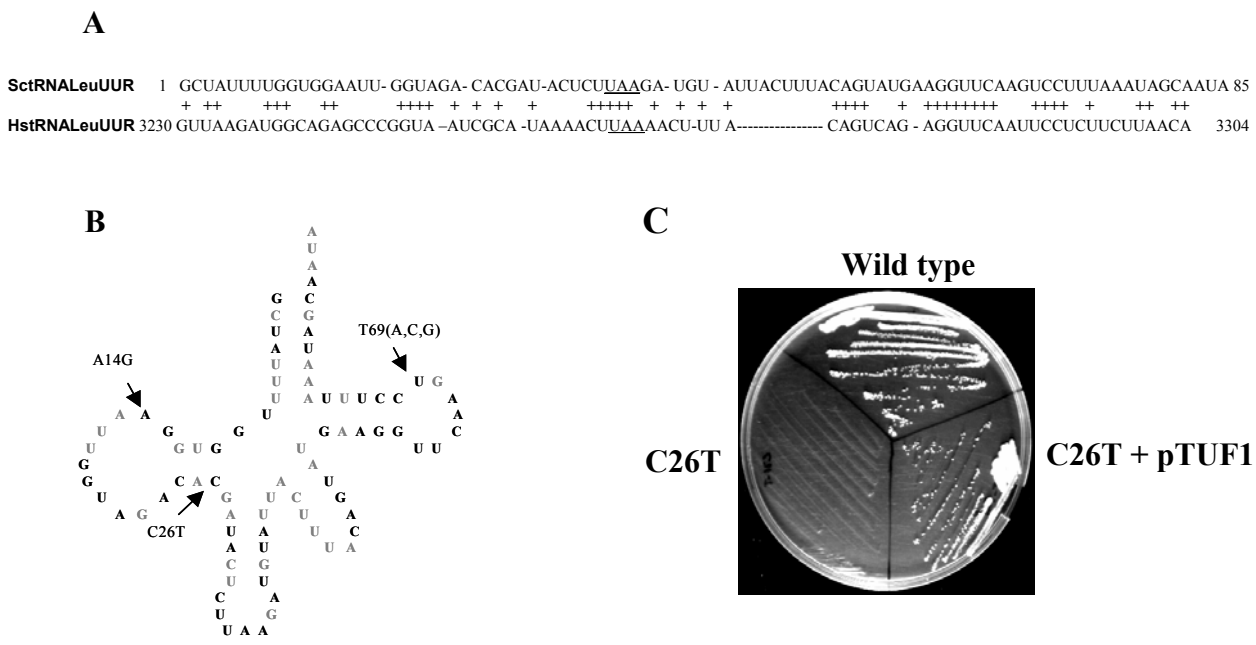


Fig. 10 Panel A shows the comparison between the yeast and human tRNALeu(UUR) sequences. Panel B shows the mutated positions in the yeast mitochondrial tRNALeu. The nucleotides coloured in grey represents the conserved bases between yeast and humans. Panel C shows the growth capability on respiratory medium of the wt strain, the strain carrying the C3256T equivalent mutation and the same mutant transformed with the multi-copy plasmid carrying the TUF1 gene.

1.11 Aims of the work

An increasing number of pathologies are associated with point mutations in mitochondrial tRNA genes but the variable phenotype and the existence of polymorphisms remain a great obstacle for the analysis in the human cells.
Table 1 reports that the majority of the mutations correlated with pathologies are distributed in tRNALeu(UUR), tRNAIle and tRNALys genes.
tRNAs are very conserved molecules because of their crucial role in the protein biosynthesis and their interactions with other cellular macromolecules. The analysis of tRNA sequences revealed in some cases an high percentage of conserved nucleotides between yeast and humans. In relating a list of human pathogenic mutations to the sequence of their yeast orthologs, it has been noted that some conserved positions may have the same structural and functional importance; therefore these positions were selected to be object of further investigations.
The results obtained with the mitochondrial mutants in the tRNALeu suggested that mutations, correlated in humans with neurodegenerative diseases, could be introduced and analysed in yeast. Therefore, it was decided to take advantage of the similarity between the yeast and the human tRNALys to construct a mutant in position 38 of the yeast mitochondrial tRNALys. In humans this position was reported by Houshmand *et al.* as a novel heteroplasmic point mutation consisting in a transition from G to A in position 8328 (Houshmand *et al.* 1999). The level of mutated mtDNA varied from 57 % in muscle to 10 % in lymphocytes; the muscle tissues had abnormal accumulation of mitochondria and cytochrome c oxidase-deficient fibers. This mutation was not found in the muscles or lymphocytes of the mother and the daughter of the proband, so this is the first case of a sporadic mutation in a patient expressing a mitochondrial encephalomyopathy.
Prof. M. Zeviani proposed to introduce in yeast the mutation corresponding to the T4290C, which corresponds to position 33 in the mitochodrial tRNAIle gene. In humans this mutation was reported by Limongelli and colleagues as a homoplasmic mutation present in two sisters affected by a mitochondrial disease (Limongelli *et al.* 2004). One sister was normal until the age of 16, and then developed blurred vision, diplopia, headaches, vertigo and weakness. Her symptoms increased month after month. A younger sister showed the same disorders but they started at an earlier age (6 years old) and she died at the age of 21. For both girls, the brain magnetic resonance showed bilateral necrotising encephalomyelopathy, consistent with Leigh Syndrome. Another sister died at the age of 1 year, of respiratory arrest after a minor viral infection. Her autopsy reported abnormalities in the mesencephalon. Their mother was 55 years old and had no neurological symptoms.
The characterization of two other yeast mutants isolated after random mutagenesis is also reported in this thesis. These mutants bear base substitutions in the anticodon stem of the tRNA molecule, similarly to those analysed for the biolistic mutants. The comparison of phenotypes and the structural analysis of different tRNA mutants may be helpful in understanding the importance of each single nucleotide in the functionality of this key molecule.
Moreover, these mutants were used to search for nuclear encoded proteins able to rescue mitochondrial defects. The efficiency of the mitochondrial-tRNA synthetases as well as the EF-Tu to suppress the defective phenotype was tested.
Finally, the importance of the nuclear background in which the mitochondrial mutation is expressed was investigated. With this aim different experimental approaches were tested, changing the nuclear context for each mutant and analysing the expression level of TUF1 gene in different wild-type and/or rho° strains.

Materials and methods

2.1 Strains and conditions

- *S. cerevisiae* strains:

D273-10B/A1 MATα, met$^-$ ura3, Δleu, Kan^R, rho$^+$ (Berlani *et al.* 1980)
FF1210-6C MAT α, ura 1-2, rho° and rho$^+$ (kindly provided by Prof. M. Bolotin-Fukuhara)
FF1210-6C MAT α, ura 1-2, Δleu, Can^R, Kan^R, rho$^+$ (kindly provided by Prof. M. Bolotin-Fukuhara)
MCC123 MAT a, ade2, ura3-52, kar1-1, rho° and rho$^+$ (Mulero and Fox 1993)
MCC123 MAT a, ade2, ura3-52, Δleu, kar1-1, Kan^R, rho° and rho$^+$ (kindly provided by Prof. M. Bolotin-Fukuhara)
YGM128 MAT α, ura 1-2, Δleu, Can^R, Kan^R, rho° and rho$^+$ (Bolotin-Fukuhara *et al.* 1977)
TF145 MAT α, ade2-1, ura3-52, mit$^-$ oxi1-17 (Fox *et al.* 1991)
D273-10B/A1 and FF1210-6C rho° strains have been obtained by Ethidium Bromide mutagenesis as described in par. 2.18.

- mitochondrial *syn*$^-$ mutants:
x14.25 MAT α, met$^-$ (Macino *et al*. 1979, Berlani *et al. 1980*)
U42C MAT α, met$^-$ (Berlani *et al.* 1980)
Ts9 MATα, *ura1-2, Δleu,* Kan^R (Elelj-Fridhi *et al.* 1991)
C26T MAT a, ade 2, ura 3-52, ura1-2, Δleu, Can^R, Kan^R (Feuermann *et al.* 2003)
T69C MAT a, ade 2, ura 3-52, ura1-2, Δleu, Can^R, Kan^R (Feuermann *et al.* 2003)
A14G MAT a, ade 2, ura 3-52, ura1-2, Δleu, Can^R, Kan^R (Feuermann *et al.* 2003)

- synthetic rho$^-$ (containing only the pJM2 vector with the mutated tRNA gene as unique DNA inside their mitochondria) obtained in this work:
LysG38A a, ade2, ura3-52, kar1-1
LysG38C a, ade2, ura3-52, kar1-1
IleT33A a, ade2, ura3-52, kar1-1

- biolistic mutants obtained in this work:
LysG38A a, ade2, ura3-52, kar1-1
LysG38C a, ade2, ura3-52, kar1-1
IleT33A a, ade2, ura3-52, kar1-1

- *syn*$^-$ mutants obtained from nuclear background changes:
D/x14.25 MATα met$^-$, ura3, Δleu, Kan^R
M/ x14.25 MAT a ade2, ura3-52, Δleu, Kan^R, kar1-1
F/ x14.25 MATα ura 1-2, Δleu, Can^R, Kan^R

D/U42C MATα met$^-$, ura3, Δleu, Kan^R
M/ U42C MAT a ade2, ura3-52, Δleu, Kan^R, kar1-1
F/ U42C MATα ura 1-2, Δleu, Can^R, Kan^R

D/ Ts9 MATα met$^-$, ura3, Δleu, Kan^R
M/Ts9 MAT a ade2, ura3-52, Δleu, Kan^R, kar1-1

D/LysG38A MATα met$^-$, ura3, Δleu, Kan^R
F/Lys G38A MATα ura 1-2, Δleu, Can^R, Kan^R

- *E. coli* strain:

DH5α: end A, hsd R17, sup E44, thi-1, recA1, gyrA, relA1 D (lacZYA-argF), U169 (F80lacZ ΔM15).

- Yeast growth media:

- Yeast Extract Peptone (YP): 1 % yeast extract and 1 % peptone, supplemented with: 2 % glucose (YPD), 2 % galactose, 3 % glycerol, 6 % ethanol, 2 % lactate or 0.4 % galactose 3 % glycerol.

- Minimum media, 10X Yeast Nitrogen Base (WO) (6.7 g in 100 ml) 2 % glucose, supplemented by the required amino acids (50X,1 mg/ml).

- *E. coli* growth media:

- LB: 1 % tryptone, 1% NaCl, 0.5% yeast extract, supplemented with ampicillin (1 mg/ml) when required.

Solid media contain 1.5 % agar.

2.2 Oligonucleotides

- PCR and sequence oligonucleotides:

5'Thr1^{+}: 5'-CCCTATTTAAGAAGGAGTTT-3'
Val^{-}: 5'-GGTGTCTATATATAGGTTTTG-3'
Arg2^{+}: 5'-CTAATTATATAGGTTCAAATCC-3'
Tyr2^{-}: 5'-GAAGGGAATAGGAATTGAAC-3'
Leu^{+}: 5'-GCTATTTTGGTGGAATTGG-3'
Arg1^{-}: 5'-ATATTAGAAGTATTATGCTTTAAC-3'

- Oligonucleotides utilized for the site directed mutagenesis:

LysG38A^{+}: 5'-CAGTTGTTTTAAACAACCCATGCTTG-3'
Lys G38A^{-}: 5'-CCAAGCATGGGTTGTAAAAGACAACTG-3'
LysG38C^{+}: 5'-CAGTTGTTTTAACCAACCCATGCTTG-3'
Lys G38C^{-}: 5'-CCAAGCATGGGTTGGAAAAGACAACTG-3'
LysG38T^{+}: 5'-CAGTTGTTTTAATCAACCCATGCTTG-3'
LysG38T^{-}: 5'-CCAAGCATGGGTTGAAAAAGACAACTG-3'

IleT33C^{+}: 5'-GGTTAGAATAGTATTCTGATAAGGTACAAATATAGG-3'
IleT33C^{-}: 5'-CCTATATTTGTACCTTATCAGAATACTATTCTAACC-3'
IleT33A^{+}: 5'-GGTTAGAATAGTATTATGATAAGGTACAAATATAGG-3'
IleT33A^{-}: 5'-CCTATATTTGTACCTTATCATAATACTATTCTAACC-3'
IleT33G^{+}: 5'-GGTTAGAATAGTATTGTGATAAGGTACAAATATAGG-3'
IleT33G^{-}: 5'-CCTATATTTGTACCTTATCACAATACTATTCTAACC-3'

- 5' –end labelled probes:

THR1: 5'-CCTAAAAACATACATTTTACC-3'
ILE: 5'-CCTATATTTGTACCTTATC-3'
ARG1: 5'-ATATTAGAAGTATTATGCTTTAAC-3'
ASP: 5'-CCTCCATTATGACAAAATGG-3'
GLN: 5'-GAATCGGTTTGATTCGAACA-3'
LEU: 5'-GTAATACATCTTAAGAGTATCG-3'
VAL: 5'-GGTGTCTATATATAGGTTTTG-3'

- TUF1 oligonucleotides:

$TUF7^{+}_{NOT}$: 5'-TTGCGGCCGCAATTGTAAACTATTTTGTGCT-3'
$TUF4^{-}_{NOT}$: 5'-TTGCGGCCGCAAGAAATGAACAGAATATATAG-3'
$TUF1^{+}$: 5'-GTGGTATTACAATTTCTACTG-3'
$TUF5^{-}$: 5'-GATAGCACCATCCATTTG-3'

2.3 Vectors

- TUF1 containing vectors:

- YEpLAC181TUF1 (Rinaldi *et al*., 1997) also denominated p181TUF1
- pFL61TUF1 in which the TUF1 gene, with its own promoter, recleaved from the YEpLAC181TUF1, is cloned into EcoRI restriction site of pFL61 vector (Bonneaud *et al.* 1991)
- $pFL61TUF1_{NOT}$ in which the TUF1 gene is cloned into NotI restriction site of pFL61

In order to obtain $pFL61TUF1_{NOT}$, the TUF1 gene was amplified using $TUF7^{+}_{NOT}$ and $TUF4^{-}_{NOT}$ oligonucleotides containing the NotI restriction site (Fig. 11).

- Aa-tRNA-synthetase gene containing vectors:

- pCNAM2 (YECpGMC068 Herbert *et al.* 1988) in which the NAM2 gene is cloned into the centromeric vector pFL39 (Bonneaud *et al.* 1991) under the GAL promoter

- pENAM2 (kindly provided by Prof Claude Jacque) in which the NAM2 gene, with its own promoter, is cloned into the multi-copy vector pFL44 (Bonneaud *et al.* 1991)
- pMSK1 (kindly provided by Prof. Ivan Tarassov) in which the MSK1 gene, with its own promoter, is cloned into pRS416 (Sikorski and Hieter 1989)

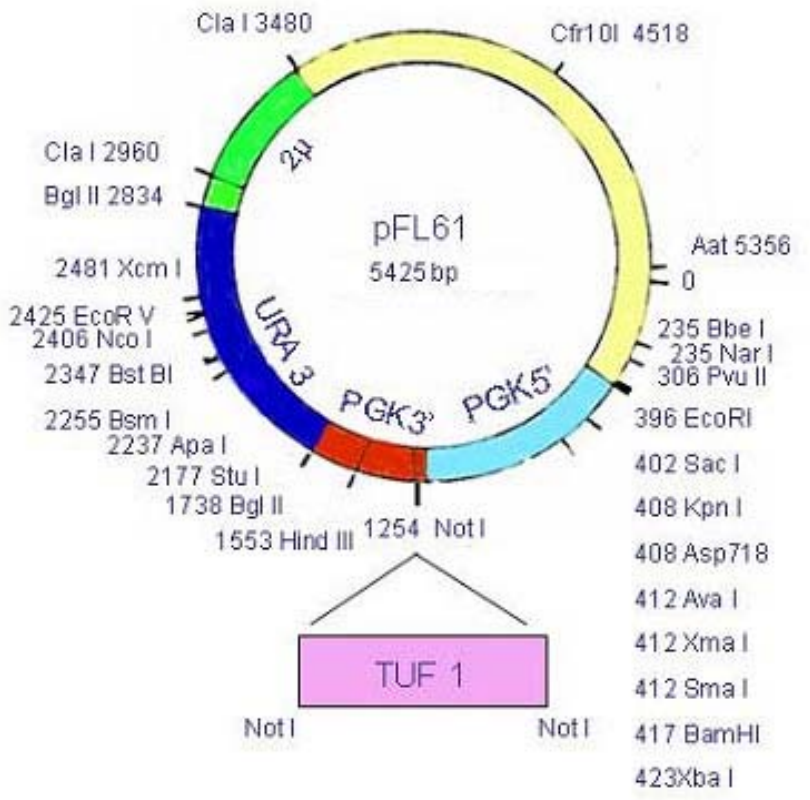


Fig. 11 Restriction map of pFL61 vector. TUF1 gene was cloned into NotI restriction site under the PGK1 promoter.

- Biolistic transformation vectors:

- pTZ18 (Biorad)
- pKS (pBluescriptII KS, Stratagene)
- pJM2 derived from pTZ18 and containing the OXI1 gene (Mulero and Fox 1993)
- Yep352 (Hill *et al.* 1986)
- pJMLysG38A in which the 1250 bp mutated tRNALys gene containing fragment is cloned into pJM2 vector
- pJMLysG38C in which the 1250 bp mutated tRNALys gene containing fragment is cloned into pJM2 vector
- pJMIleT33A in which the 800 bp mutated tRNAIle gene containing fragment is cloned into pJM2 vector

2.4 Mitochondrial DNA miniprep

2 ml YP 2 % galactose cultures were transferred to eppendorf tubes, centrifuged and washed with 1 ml of distilled water. The pellet was resuspended in 0.2 ml of spheroplasting buffer (1.2 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA pH 7, 2 % w/v Zymolyase-20T, 1 % v/v β-mercaptoethanol) and incubated for one hour at 37°C. 0.75 ml of lysis buffer (0.5 % SDS, 100 mM Tris-HCl pH 7.4, 50 mM EDTA pH 8) was then added. After 30 minutes at 65°C, 0.2 ml of 5 M potassium acetate was added and the mixture was further incubated in ice for one hour.
The tubes were centrifuged for 10 minutes at 13,000 rpm and 0.75 ml of supernatant was transferred to a clean new tube, taking care to avoid the surface. The DNA was precipitated with 0.75 ml of isopropanol, centrifuged for 10 seconds, resuspended in 0.3 ml of TE (10 mM Tris-HCl, 1 mM EDTA) containing RNase (30 μg/mg final) and, incubated for one hour 37°C. A second precipitation (0.4 M sodium acetate pH 7 plus 3 volumes ethanol) provided mitochondrial DNA clean enough to be restricted and cloned. The final resuspension was in 50 μl of TE pH 8.

2.5 Mitochondrial isolation and purification

200 ml of YP 2 % galactose cultures were centrifuged at 3,000 rpm for 10 minutes and washed with water twice. The pellet was resuspended in a solution containing 0.64 M β-mercaptoethanol, 0.25 % EDTA pH 7, and incubated for 20 minutes at room temperature. 0.75 ml of lysis buffer (0.5 % SDS, 100 mM Tris-HCl pH 7.4, 50 mM EDTA pH 8) was then added.
After centrifugation for 10 minutes at 2,500 rpm, the pellet was washed with a solution containing 1.1 M sorbitol, 0.05 M phosphate buffer pH 7.5 and resuspended in the same solution in which the Zymolyase-20T have been added (1mg per 1-3 g of cells). The Zymolyase digestion was incubated at 37°C and followed by O.D. measurements at 600 nm. When the O.D. measure was 10 time less than the initial value the digestion was interrupted. Protoplasts were centrifuged at 6,000 rpm for 10 minutes and washed with 1.1 M sorbitol. Protoplasts were resuspended in lysis buffer containing 0.6 M sorbitol and 10 mM Tris-HCl pH 7.4.
When mitochondria are isolated to prepare RNA extracts, 10 mM vanadylsolphate-ATP, as RNase inhibitor, is added. Protoplasts were broken using waring blendor, three times at the maximum speed for 30 seconds and then centrifuged at 2,500 rpm 10 minutes, the supernatant was centrifuged until cells or debris were completely removed.
The supernatant was centrifuged at 13,000 rpm for 15 minutes to obtain the mitochondrial pellet.
This pellet was washed three times with lysis buffer. The mitochondrial pellet could be stored at –70°C.

2.6 DNA restriction analysis

Restriction enzymes recognize DNA specific sequences and generating fragments of variable length, having blunt or protruding ends. Each enzyme requires specific condition of buffer and temperature. Digested fragments were checked on 0.7-1 % agarose gel with 1X TBE buffer (10X stock solution: 1M Tris base pH 7.4, 0.9 M boric acid, 0.01 M EDTA pH 7). The electrophoresis was conducted at 30 mA for the necessary time and the fragments were

detected with Ethidium Bromide (1 μl/ml) staining. Lambda phage digested EcoRI/HindIII was used as marker.

2.7 Total RNA extraction from purified mitochondria

The mitochondrial pellet was resuspended in 200 μl of 0.3 M sodium acetate pH 5 and 10 mM EDTA pH 5. RNAs were extracted by phenol-chloroform pH 5 two times and precipitated overnight using 0.3 M sodium acetate pH 5 and two volumes of 95 % ethanol at –20°C.
After centrifugation for 15 minutes at 13,000 rpm, the pellet of RNA was washed two times with 80 % ethanol and resuspended in 40 μl of 10 mM sodium acetate pH 5, 1 mM EDTA pH 4.5.
RNA concentration was determined by measuring the O.D. at 260 nm.
tRNA extracted in these conditions are acylated, to obtain deacylated tRNAs the RNA extract were subjected to alkaline treatement. 1M Tris base was added to the samples to the final concentration of 0.2 M and the solution was incubated for 90 minutes at 37°C.

2.8 Northern blot

Two glass plates (60x40 cm), typically one slightly different in size from the other, and spacers (0.5 mm) were used to form a watertight seal to put the unpolymerized gel solution. The glass plates were washed carefully with 95 % alcohol and the spacers were arranged at each side parallel to the two edges. The entire length of the two sides and the bottom of the plates were bound with yellow electrical tape.
To prepare the 6 % polyacrylamide/ 8 M urea gel, 32 g of urea and 10 ml of acrylamide (19:1 acryl:bisacrylamide) were mixed with 2.2 ml of 3 M sodium acetate pH 5. The solution was warmed to dissolve the urea and then water was added up to the final volume of 66.6 ml. 35 μl of TEMED (N-N-N-N-tetramethylethylendiamine) and 380 μl of APS (ammonium persulfate) were mixed to the gel solution. The gel solution was immediately introduced in the space between the two glass plates. The appropriate comb was inserted and the two glass plates were laid to minimize distortion of the gel. Acrylamide polymerize in 30 minutes at room temperature. The comb and the tape were removed and the two glass plates containing the gel were attached to the electrophoresis tank. The upper ad lower reservoirs were filled with 0.1 M sodium acetate pH 5.
8-10 μg of RNAs were mixed with BBF (bromophenol blue and xylene cyanol) dyes and 8 M urea with 0.1 M sodium acetate pH 5. The electrophoresis was conducted at 300 V for 20 hours at 6°C. In these not completely denaturing conditions RNA molecules are separated depending on their secondary structure and length.
After the electrophoresis the two glass plates were separated and the gel was washed two times with 5X SSC (20X stock solution: 3 M sodium cloride, 0.3 M sodium citrate). The gel was then transferred to nitrocellulose filters (Amersham Hybond-XL) using gel dryer for 1 hour and 30 minutes at 80°C.
RNA were fixed using UV rays for 3 minutes on each side. The acrylamide was removed and the filters were pre-hybridized with 5X SSC, 5X Denhardt's solution (100X Denhardt's: BSA2 %, 2 % ficol, 2 % polyvinyl-pyrrolidone) and 0.02 mg/ml of denatured salmon sperma.

2.9 5'-labeling of oligonucleotide primers

The following mixture was prepared on ice: 20-25 picomole of oligonucleotide; 25 μCi of ^{32}P γdATP; 5 units/μl of bacteriophage T4 polynucleotide kinase; 2 μl of kinase buffer 10X (500

mM Tris pH 9.5, 50 mM Dithiothreitol, 100 mM $MgCl_2$) and water to the final volume of 20 µl. The solution was incubated at 37°C for 30 minutes. 80µl of STE (0.1 M NaCl and TE) were added to the solution. A sephadex G-50 resin was prepared by centrifugation at 1,600 rpm for 5 minutes and equilibrated with STE. The solution containing the labeled oligonucleotide was applied to the resin and equilibrated with TE. The labeled solution was eluted and collected into an eppendorf tube and precipitated using 0.3 M NaAcetate, 3 µl of 5 mg/ml tRNA carrier and three volume of 95 % ethanol.

2.10 DNA-RNA hybridization

The 5'-end labeled probe was denaturated at 90°C and added to the pre-hybridized filter containing the RNAs. The hybridization was conducted for 12 hours at the same temperature as the pre-hybridization.
After hybridisation, the filter was removed from the solution and washed two times with a solution containing 2X SSC, 0.1 SDS for 15 minutes at the same temperature. The filter was then washed one time with a solution containing 1X SSC, 0.1 SDS for 30 minutes. The filter was dried and exposed for 24-48 hours to Biomax MR (Kodak) at –70°C.

2.11 Polymerase Chain Reaction (PCR)

Two oligonucleotide primers were used to amplify specific sequences of DNA using the Polymerase Chain Reaction technique (PCR). To conduct the reaction, a mixture of 100 µl was prepared as follow: 1 µg of template DNA, 10 µl of 2 mM $MgCl_2$, 0.2 µM of each primers, 10 µl of 10X PCR buffer (10 mM Tris-HCl pH 9, 50 mM KCl, 0.2 ng/ml BSA). This mixture was denaturated for 5 minutes at 95°C and placed immediately on dry-ice plus ethanol; subsequently, the reaction mixture was placed at the annealing temperature of 56°C. 2 µl of dNTP (0.2 mM) and 0.5 µl of Taq DNA polymerase 2.5 U were added to the mixture. The amplification was carried out starting from 2 minutes at 56°C, continuing for 3 minutes at 72°C for polymerisation, and again at 94°C for 1 minute for denaturation. These conditions were repeated for 44 cycles. 6 µl of PCR products were checked on agarose gel.

2.12 DNA fragments extraction from agarose gels

The DNA was loaded on 1% low melting agarose gel in TA (0.04 M Tris-acetate). After the electrophoresis the band containing the fragment of interest was cut and saved into an eppendorf tube. The band was purified using the Amersham kit for gel extractions. The sample was eluted using 50% TE.
The sequence analysis was determinated by Bio Molecular Research Sequencing Service (BMR - Padova).

2.13 Preparation of competent *E. coli* cells

A single colony of *E. coli* DH5α was inoculated in 2 ml of LB medium and incubated overnight at 37°C. 400 µl of this culture were inoculated in 40 ml of LB medium until the concentration of 0.2-0.3 OD measured at 550 nm. This culture was incubated on ice for 20 minutes and then centrifuged at 3,500 rpm for 5 minutes. The pellet was resuspended in 20 ml of a sterile ice-cold sterile solution containing 0.05 M $CaCl_2$. After 30 minutes on ice the cells were centrifuged and resuspended in 4 ml of the solution above described and incubated for 3-4 hours. This treatment causes cell wall modifications which increase the permeability of the

cells to exogenous DNA. The cell pellet was resuspended in ice-cold sterile glycerol/$CaCl_2$ solution (glycerol, 15 % (v/v); 50 mM $CaCl_2$) and stored in 300 µl aliquots at –70°C.

2.14 Transformation of *E. coli*

40-50 ng of DNA were added to 300 µl of competent *E. coli* cells. The mixture was incubated for 40 minutes on ice. After an heat shock of 3 minutes at 42°C, which allows the exogenous DNA to enter the cells, the mixture was incubated for 10 minutes at room temperature. 1 ml of LB was added, and the cells were incubated at 37°C for 30-60 minutes.
200 µl of the mixture containing the transformed cells were plated on LB plus ampicillin, to select the transformants.

2.15 Miniprep of *E. coli* pDNA

1.5 ml of an E. coli overnight culture was centrifuged and resuspended in 100 µl of a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8. After 5 minutes at room temperature 200 µl of a fresh solution, containing 0.2 M NaOH, 1% SDS, were added. After 5 minutes in ice, 150 µl of ice-cold high salt buffer (3M K-Acetate 5 M Acetic Acid) were added and the mixture was incubated for 5 minutes at 0°C. After 5 minutes of centrifugation at 13,000 rpm, two volumes of 95 % ethanol were added and the mixture was centrifuged for 10 minutes. The pellet was washed with 70 % ethanol, dried and resuspended in 30 µl of 1X TE containing RNase (20 µg/ml).
2 µl were checked on agarose gel. This preparation of DNA can be stored at –20°C.

2.16 Maxiprep of *E. coli* pDNA

To obtain large amount of *E. coli* purified plasmid DNA (pDNA), a colony from transformed cells was inoculated in 50 ml of LB ampicillin and incubated overnight at 37°C. The pDNA was extracted following the instructions of the Qiagen HiSpeed Plasmid Maxiprep Kit manual. The protocol is based on a modified alkaline lysis procedure, followed by binding of DNA to Qiagen anion-exchange resin on appropriate low-salt and pH conditions.
The pDNA was then simply eluted from the resin with TE PH 7.4.

2.17 Transformation of *S. cerevisiae* using LiCl procedure

Yeast cells were grown in 100 ml of YPD medium to $5x10^6$-$2x10^7$ cells/ml (log phase). The pellet obtained after centrifugation for 5 minutes at 3,000 rpm was washed with 10 ml of TE and resuspended in 1ml of TE containing 0.1 M LiCl. After 60 minutes at 30°C, 100 µl of cells were transferred into eppendorf tubes. 5 µl of 10 mg/ml salmon sperm DNA, as a carrier, and the appropriate amount of pDNA (2-10 µg) were added to the cells. To one aliquot of cells only the carrier DNA was added (control). After an incubation of 30 minutes at 30°C, 1 ml of 40 % PEG 4000 was added and the mixture was incubated for 60 minutes at 30°C.
5 minutes at 42°C provided the heat shock necessary to allow the pDNA to enter the cells. The cells were then centrifuged for 50 seconds at 3,000 rpm and washed with water twice.
300 µl of YPD was added to the pellet and the cells were plated on selective media.

2.18 Ethidium Bromide mutagenesis

Yeast cells, grown to stationary phase, were diluted to a concentration of $5x10^6$-$2x10^7$ in 2.5

ml YPD media. 0.25 ml of 1 M phosphate buffer pH 6.5 and 12 µl of 10 mg/ml ethidium bromide were added to the cells and the mixture was incubated overnight. The cells were then plated as single colonies on YPD and replica plated on YP 3 % glycerol media to verify the absence of growth due to the loss of mitochondrial DNA.

2.19 Small scale yeast total RNA isolation

About $4x10^8$ yeast cells were centrifuged, washed with 1 ml of H_2O and transferred into an eppendorf tube. The pellet was resuspended in a solution containing: 0.5 M NaCl, 0.2 M Tris/HCl pH 7.5, 0.01 M EDTA pH 7.5 and 1 % SDS. 0.4 g of glass beads (0.25-0.3 mm or 0.45 mm) were added to the solution. 0.2 ml of phenol-chloroform isoamylalcohol (25:24:1)(PCI) were added to the solution and vortex at top speed for 2'-2'30''. After 10 minutes of centrifugation the aqueous phase was removed and re-extracted in a new tube with 0.3 ml more PCI.
The RNA was precipitated overnight at –20°C using 0.3 M sodium acetate pH 5 and two volumes of 95 % ethanol. After centrifugation for 15 minutes at 13,000 rpm, the pellet of RNA was washed two times with 80 % ethanol. The pellet was dissolved in sterile water.
RNA concentration was determined by measuring the O.D. at 260 nm.

2.20 RT-PCR Analysis

Total RNAs were treated with RNase-free DNaseI (Roche Diagnostic GMbH, Mannheim, Germany), and their purity and integrity were confirmed by visualisation after separation by electrophoresis on agarose gel.
2 µg of treated RNAs were used to perform a Reverse Transcriptase Reaction, using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) by Promega. A solution containing the RNAs and 0.5 µg of random hexamers per µg of mRNA sample in a total volume of 15 µl of water was incubated at 70°C for 5 minutes and then cooled immediately. To this solution were added: 8 µl of 5X reaction buffer (250 mM Tris-HCl pH 7.4, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 5 µl of 5 mM dNTP, 0.63 µl of rRNasin Ribonuclease inhibitor, 1 µl of M-MLV RT, and water to final volume of 25 µl. The solution was incubated for 60 minutes at 37°C followed by 2 minutes at 94°C for denaturation of the reverse transcriptase. 2 µl of cDNA were used to perform the Real Time Polymerase Chain Reaction (RT-PCR).
To identify the concentration of cDNA (unknown samples) using the RT-PCR technique, a commercial vector containing the gene of interest was carefully quantified and used to prepare standard samples of a known quantity (Guenzi 2004). The standard samples were prepared by adding to the serial dilutions of DNA (from 1.6 10^{-2} to 2.56 10^{-5} ng), 25 µl of the reaction mixture, containing 1X iQ SYBR Green Supermix (BioRad), 0.3 mM oligonucleotides, and H_2O to the final volume of 50 µl. Each standard sample was prepared in duplicate. The unknown samples were prepared with 50 ng of cDNA and the same reaction mixture as the standard samples; the unknown samples were prepared in triplicate.
Amplification was carried out in the iCycler apparatus from Biorad, as follow: a denaturation step of 5 minutes at 95°C, and 35 cycles of 95°C for 30'', 54°C for 30'', and 72°C for 45''.
Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescence signal is first recorded as statistically significant above background (Gibson *et al.* 1996). This point is defined as the Ct, and will always occur during the exponential phase of amplification. The reporter signals were analysed using the iCycler iQ software (BioRad).

These values can be translated into a quantitative result by constructing a standard curve, with the standard sample values.
A melting curve was obtained after completion of the cycles to verify the presence of a single amplicon. PCR samples were subjected to a stepwise increase in temperature from 54°C to 95°C and fluorescence measurements were taken at every temperature increment. The melting products cause SYBR green dissociation resulting in decreased fluorescence. If primer-dimer or nonspecific products were made during the amplification step, they will generally melt at a lower temperature (Tm) than the desired products and in the melting curve two peaks will appear.

2.21 Site directed mutagenesis

The site directed mutagenesis was performed following the instruction manual of the QuickChange site-directed mutagenesis kit by Stratagene. This kit was used to introduce point mutations in specific genes. For this procedure it was utilized 10 ng of double-stranded DNA vector containing the insert with the desired wild-type gene and two synthetic mutagenic primers complementary to opposite strands of DNA, containing the desired mutation. The samples were prepared by adding to the template DNA, 125 ng of each oligonucleotide, 1 µl of dNTP mix, 5 µl of reaction buffer, 1 µl of Pfu Turbo polymerase (2.5 U/µl) and water to the final volume of 50 µl. The oligonucleotides were extended during PCR cycling by using Pfu Turbo DNA polymerase (5' at 95°C followed by 18 cycles: 1' 95°C, 1'30'' at 56°C and 9' at 68°C, plus an extension of 7' at 68°C). The product was treated by 1 µl DpnI. This enzyme digested the methylated and hemimethylated parental DNA. The resulted product had only the mutation-containing new synthesized DNA. This DNA was used to transform competent *E. coli* DH5α cells according to the transformation protocol reported in par. 2.14.

2.22 Yeast biolistic transformation

This transformation was performed as described by Bonnefoy and Fox 2000.
Cell preparation:
The rho° strain to be bombarded was grown for 2-3 days (stationary phase) in 2 % galactose/0.1 % glucose. 30 ml of YPD medium were inoculated with 1/50 dilution of the stationary culture. One hour before the bombardment the cells were centrifuged and harvested in 1.2 ml of YPD. 100 µl of this suspension were plated on minimal medium supplemented with amino acids to provide the appropriated prototrophic selection.
Preparation of microprojectiles and precipitation of DNA:
0.7 µm of tungsten particles available from BioRad were sterilized by suspension in 1.5 ml of 70 % ethanol in an eppendorf tube and incubated at room temperature for 10 minutes. The particles were washed with 1.5 ml of sterile water and resuspended in sterile 50 % glycerol.
In an eppendorf tube 5 µg of plasmid carrying the nuclear marker and a nuclear replication origin, and 15 µg of plasmid carrying the mitochondrial DNA of interest were mixed to 100 µl tungsten particles. 4 µl of 1 M spermidine free base and 100 µl of ice-cold 2.5 mM $CaCl_2$ were added and the mixture was incubated for 10 minutes on ice with occasional vortexing. The mixture was spin briefly, 15'' at 13,000 rpm, and the supernatant was removed. The particles were resuspended in 200 µl of 100 % ethanol and the procedure was repeated until the particles were resuspended easily. The particles were resuspended in 60 µl of ethanol and distributed at the center of six macrocarriers placed in their holders, allowing the ethanol to evaporate.
Bombardment:

The experiment was done using a 1350 psi rupture disk. The open petri plate carrying the lawn of cells was placed at 5 cm from the macrocarrier loaded in its holder into the assembly system. The vacuum chamber was evacuated until 27 mmHg and the particles were shot.
Any fragments of the macrocarrier disk were removed and the plate was incubated at 28°C for 4-5 days until colonies appeared.
Identification of the mitochondrial transformants:
The plate containing the bombarded cells was replica plated on a lawn of the tester strain (TF145) containing a mit^- (oxi1-17) mutation on YPD. The cross was incubated at 28°C for 2 days. This plate was replica plated on YP 3 % glycerol media. In glycerol containing media, only cells in which the mit^- mutation is complemented by the mitochondrial marker present on one of the two shot vectors are able to grow by respiration. From the bombarded plate were picked off colonies corresponding to the positions of respiring cells. This test was repeated at least three times before pure stable synthetic rho^- clones were obtained.
The synthetic rho^- were crossed with a rho^+ strain to allow recombination between the mutated gene and the wild-type gene present on the mitochondrial genome. If one of the two strains carries the karyogamy-defective mutation Kar1-1, the nuclear fusion is reduced while the mitochondrial fusion is still efficient. This strategy was used to obtain haploid mitochondrial mutants.

2.23 DAPI staining

To visualize the DNA, 1% formaldehyde was added to 100 µl of an exponential culture of yeast. After 30 minutes at room temperature the pellet was washed with 100 µl of water. The pellet was resuspended in 50 µl of a solution containing 1 µg/ml of 4'-6-Diamidino-2-phenylindole (DAPI). This dye is known to form fluorescent complexes with natural double-stranded DNA. After an incubation of 5 minutes the cells were observed using a fluorescence microscope at the wavelength of 350 nm.

Results

3.1 Construction of new specific mitochondrial tRNA mutants

3.1.1 tRNALys G38A and G38C mutants

The mitochondrial tRNALys gene shows 43 % of conserved bases between yeast and humans. Fig. 12 shows the sequence alignment of yeast and human tRNALys genes and the secondary structures of the molecules. The human G8328 position (Houshmand *et al.* 1999) corresponds in yeast to position 38. In order to analyse the specific effect of mutations in that position, it was decided to introduce not only the G to A (the mutation correlated in humans with the Encephalopathy), but also the other two possible substitutions, G to C and G to T.

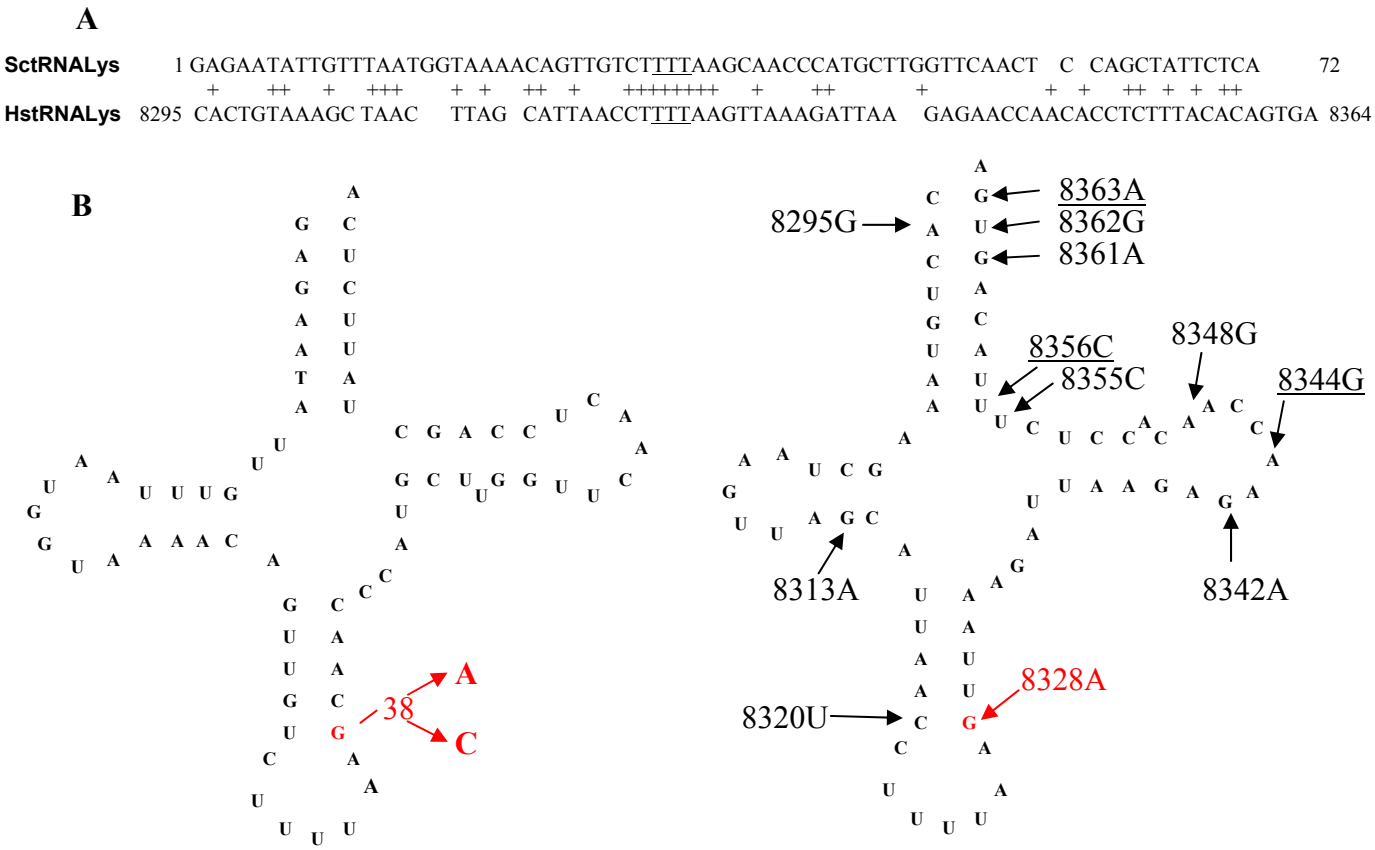


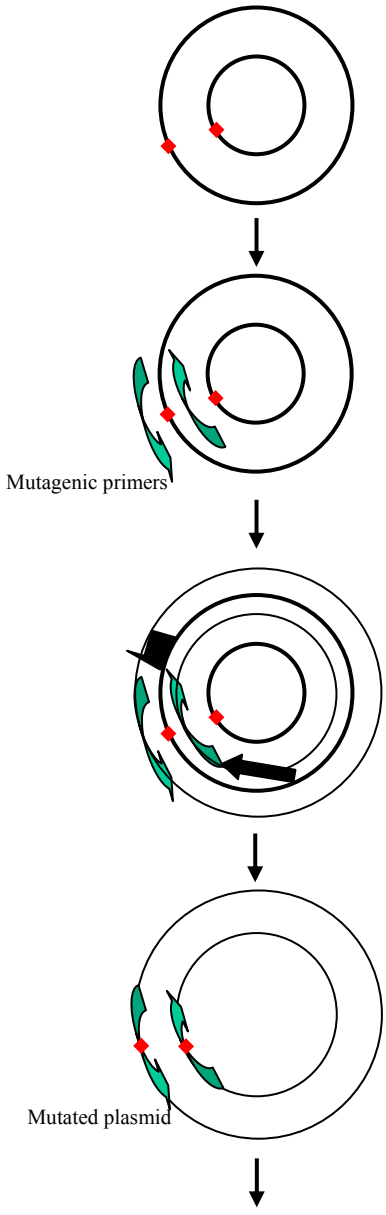
Fig. 12 Comparison of yeast and human tRNALys sequences and secondary structures. Panel A shows the alignment between the tRNA gene sequences. Plus signs indicate the conserved positions between the two genes. The anticodon is underlined. Panel B represents the yeast (left) and human (right) cloverleaf structure. In the yeast molecule are indicated the mutations studied in this thesis. In the human molecule the arrows indicate the positions correlated with pathologies (www.mitomap.org). The underlined positions indicate the mutations that have been confirmed as pathological. The red colour indicates the position analysed in this work.

From purified mtDNA of wild-type strain FF1210-6C (α, ura1,2), the TaqI fragment of 1250 bp (Fig. 13) containing tRNALys gene was cloned into ptz18 vector at the AccI restriction site.

```
66231                                   AGTTACCAAA ATTTGAGTTT   GLN
66251  GGAGTTTGTT TGTTCGAATC AAACCGATTC AATATTATAA TATATATATT
66301  ATTTATATAT AAATATATAA TTATACTCCT ATTTTTATAT TAATTAATTA
66351  ATAATATATG ATAATATAAA AATTATTGAA TTATTAACTC TTATTAATAA
66401  TAATAATAAT CATAATAATA ATATATATAT ATATAGTATA TATATAAAAG
66451  TTTTATTATA TTATATTATA TTATATATTT ATTTATATAT AATTCTTATT
66501  AATTGAAAAA AGAATAATTA ATAATCTTAT TAAAAAAATA AATACTTTCA
66551  TTTTATTTTA TTTTATTTAA TTTAATTATA ATATATAAAT ATTAAAAAAA
66601  GGATATAAGT TTTTTATAAG ATATAATATA TATATATATT AAATATAAAG
66651  AAGTTAATAT TTATATTTTA ATTATAAAAT GTTAATACTC CTTTGGGGAC
66701  TTATTAATTA AATTATTAAT TAATAATAAT TTATGATTTA TAAATAATAA
66751  ATAAAGGAAT AAGTATCAAT TAATTAATAT ATTATATTTA ATATTTTATA
66801  TTTAATATTT AATATTTAAT ATTTTAAGTT CCGGGGCCCG GCCACGGGAG
66851  CCGGAACCCC GAAAGGAGTA GTATTAATTA TGGATAGTGA GGGTGGATTT
66901  AATCCTTTTG TTATGTTATT AATTAATTAA TTAATTTATA TATATAAAAT
66951  ATTTTAATTA ATTTTTATAT AAATATATAT ATATATATAT ATTAATAATA
67001  GTCCGGCCCG CCCCGTGGGG CGGACCCCAA AGGAGTAATA TATATTATGT
67051  ATAAACAATA GAGAATATTG TTTAATGGTA AAACAGTTGT CTTTTAAGCA   LYS
67101  ACCCATGCTT GGTTCAACTC CAGCTATTCT CATAATATTA TATATATATA
67151  TTTCCCTTTC TAAAAATAAT AATAATTATA TATAATAATA ATATAATTAT
67201  ATATATATAT ATTATAATAA TAATAATAAT AATAATAATA AATAATAATA
67251  ATTATTTTTA TTAATAATAT TAATATATTA TAATTATTAA TAAATATTAA
67301  TAAAAATAGC TCTCTTAGCT TAATGGTTAA AGCATAATAC TTCTAATATT   ARG1
67351  AATATTCCAT GTTCAAATCA TGGAGAGAGT AATTATATTA TATTAATAAT
67401  CCCCCCCCCA TTTTTAATTA AATTAAGAAG TTTAATTTAC TATTTAATAA
67451  TAAATGAAAT AATAATAATA GATATAAGTT AATTGGTAAA CTGGATGTCT   GLY
67501  TCCAAACATT GAATGCGA
```

Fig. 13 Region of mitochondrial DNA containing tRNALys gene cloned in the pJM2 biolistic vector. The tRNA gene of interest is indicated in red, while other tRNA genes are indicated in green.

The wild-type sequence of tRNALys was checked by sequencing the insert of this construct using the commercial primer M13Reverse.
This plasmid was mutagenized *in vitro* following the procedure described in the Quick-ChangeTM Site-Directed Mutagenesis Kit from Stratagene (Fig. 14). Two mutagenic primers complementary to residues from 24 to 52 of tRNALys gene were used to introduce the G38A mutation (LysG38A$^+$ and LysG38A$^-$). Similar primers were used to introduce the G38C and G38T substitutions (LysG38C$^+$, LysG38C$^-$ and LysG38T$^+$, LysG38T$^-$). Only two substitutions were obtained and purified plasmid preparations were sequenced to confirm G to A and G to C mutations in position 38.
The biolistic transformation (Fig. 15) was performed as described by Rohou *et al.* 2001: each DNA fragment containing one specific mutation in the tRNALys gene was subcloned into pKS vector (pBluescriptII KS, Stratagene) digested EcoRI and SmaI. The inserts were then recleaved by EcoRI and BamHI to be ligated into the pJM2 vector (Mulero and Fox, 1993); these final vectors contain the OXI1 gene, as mitochondrial marker, and the mutated tRNALys fragment, pJMLysG38A or pJMLysG38C.
For the biolistic transformation cells from the wild-type strain MCC123 (a, ade2, ura3-52, kar1-1, rho°)(Mulero and Fox 1993) were prepared according to the method described by Fox *et al.* 1988, and Bonnefoy and Fox 2000. These cells were bombarded randomly by a large number of tungsten particles carrying two plasmids, the Yep352 (Hill *et al.* 1986) with the URA3 marker and a nuclear replication of origin, and the pJMLysG38A (or pJMLysG38C). The first plasmid allows transformants to grow on selective media (without uracile) for the presence of the genetic marker. Further genetic tests were necessary to identify the mitochondrial transformants, which received also the second plasmid in the mitochondria. The crossover between the URA$^+$ transformants with TF145 (α, ade2-1, ura3-52, mit- oxi1-17)(Fox *et al.* 1991) was performed. Both the parental strains are unable to grow on respiratory substrates (TF145 because of the presence of the oxi1-17 mutation and MCC123 rho° because of the absence of the mtDNA). Complementation events between the biolistic transformed strain and TF145 result in clones able to grow on respiratory substrates such as glycerol, in which the oxi1-17 mutation is complemented by the OXI1 allele carried by plasmid pJMLys present in the transformed cells. The corresponding haploid biolistic transformants of these positive clones were called "synthetic rho$^-$", these cells contained only the pJMLysG38A (or the pJMLysG38C) plasmid as unique DNA inside their mitochondria.
To obtain the mitochondrial recombinants, the selected transformants (denominated synthetic rho$^-$) were crossed with the wild type YGM128 (α, ura1-2, Δleu, Kan^R, Can^R)(Bolotin-Fukuhara *et al.* 1977). Around 3,500 single colonies were plated on glucose containing media. From the observation of the size of the colonies it is possible to obtain information on the mitochondrial functionality. To screen for respiratory defects, replica plating onto 3 % glycerol media (non fermentable media) at 28°C and 37°C was performed. After the screening, 18 colonies of the LysG38A showed a thermo sensitive phenotype on glycerol, growing only at 28°C. For LysG38C, 12 colonies were selected for their thermo-sensitive defective phenotype, showing slower growth at 28°C and no growth at 37°C.



Gene in plasmid with target site for mutation

Annealing of the oligonucleotide primers containing the desired mutation

Pfu Turbo DNA polymerase extend and incorporate the mutagenic primers

Digestion of the methylated, non mutated parental DNA template with DpnI

Tranformation of competent *E. coli* cells

Fig. 14 QuikChangeTM site-directed mutagenesis.

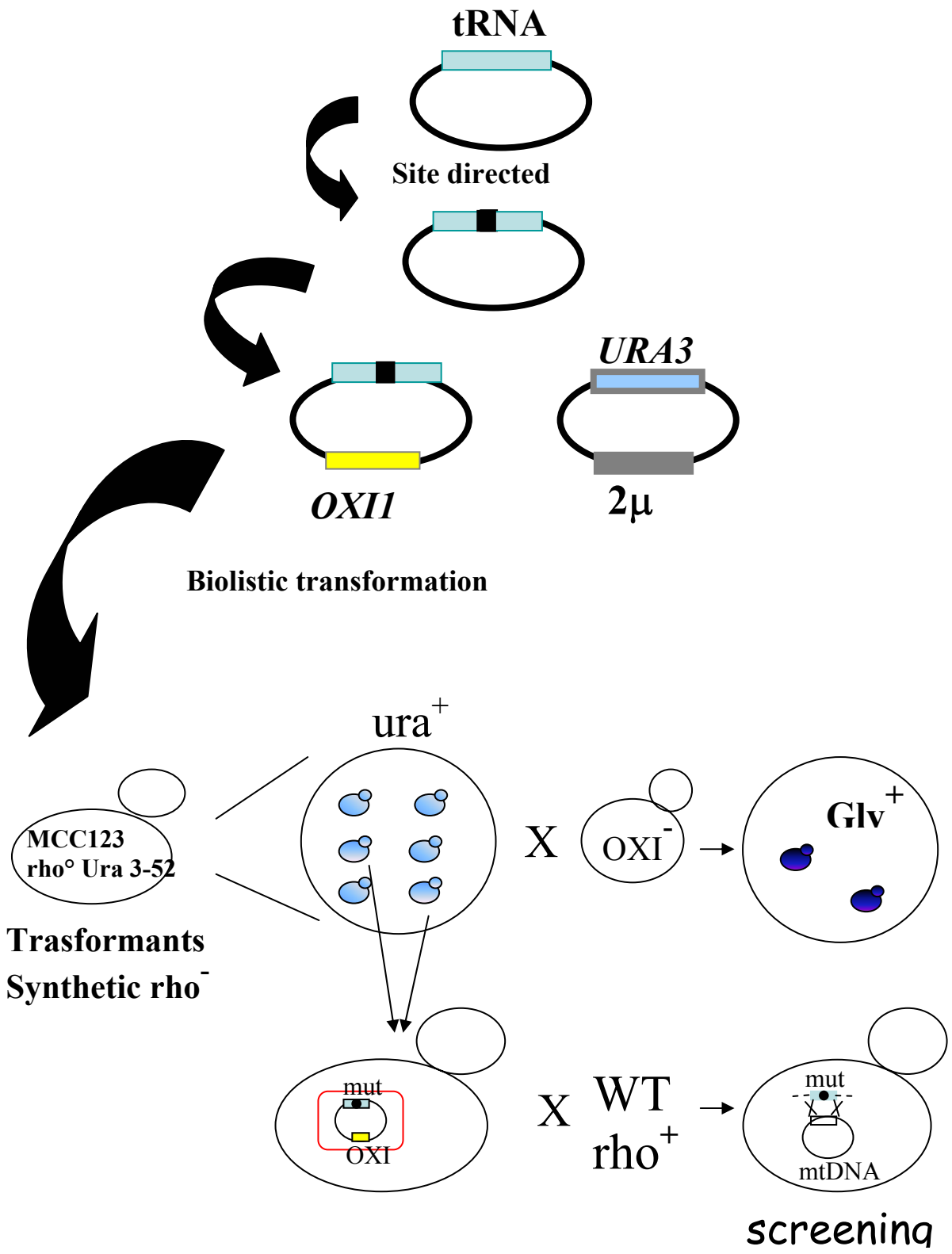


Fig. 15 Biolistic transformation.

3.1.2 tRNAIle T33A mutant

The primary structure of the mitochondrial tRNAIle gene is extremely conserved among mammals (Fig. 16). The *S. cerevisiae* tRNAIle gene has 66 % of conserved nucleotides with the human one. In order to analyse in *S. cerevisiae* mitochondrial tRNAIle the effect of the mutation correlated in humans to the MELAS Syndrome by Prof. Massimo Zeviani (Limongelli *et al.* 2003), the yeast position 33, equivalent to the human position 4290, was mutated to introduce the three possible substitutions.
MtDNA from wild-type strain FF1210-6C was digested by the NsiI restriction enzyme and a fragment of 800 bp (Fig. 17) was cloned into ptz18 vector in the PstI site.
Site-Directed Mutagenesis was performed using for each substitution two couple of mutagenic primers complementary to residues from 18 to 54 of tRNAIle gene (IleT33A^{+}, IleT33A^{-}, IleT33C^{+}, IleT33C^{-}, IleT33G^{+} and IleT33G^{-}). Only two substitutions were obtained the IleT33C and IleT33A. Purified plasmid DNA from the mutagenesis were prepared and sequenced to confirm the point mutations.
The insert containing the IleT33A mutation was subcloned into pKS vector followed by ligation to pJM2 vector, as described for the tRNALys mutants, to construct the pJMIleT33A.
After the biolistic transformation the synthetic rho^{-} was crossed with YGM128 and 4,500 single colonies were screened for their mitochondrial functionality. 30 hypothetical recombinant colonies were selected for their growth defects on glycerol containing media.

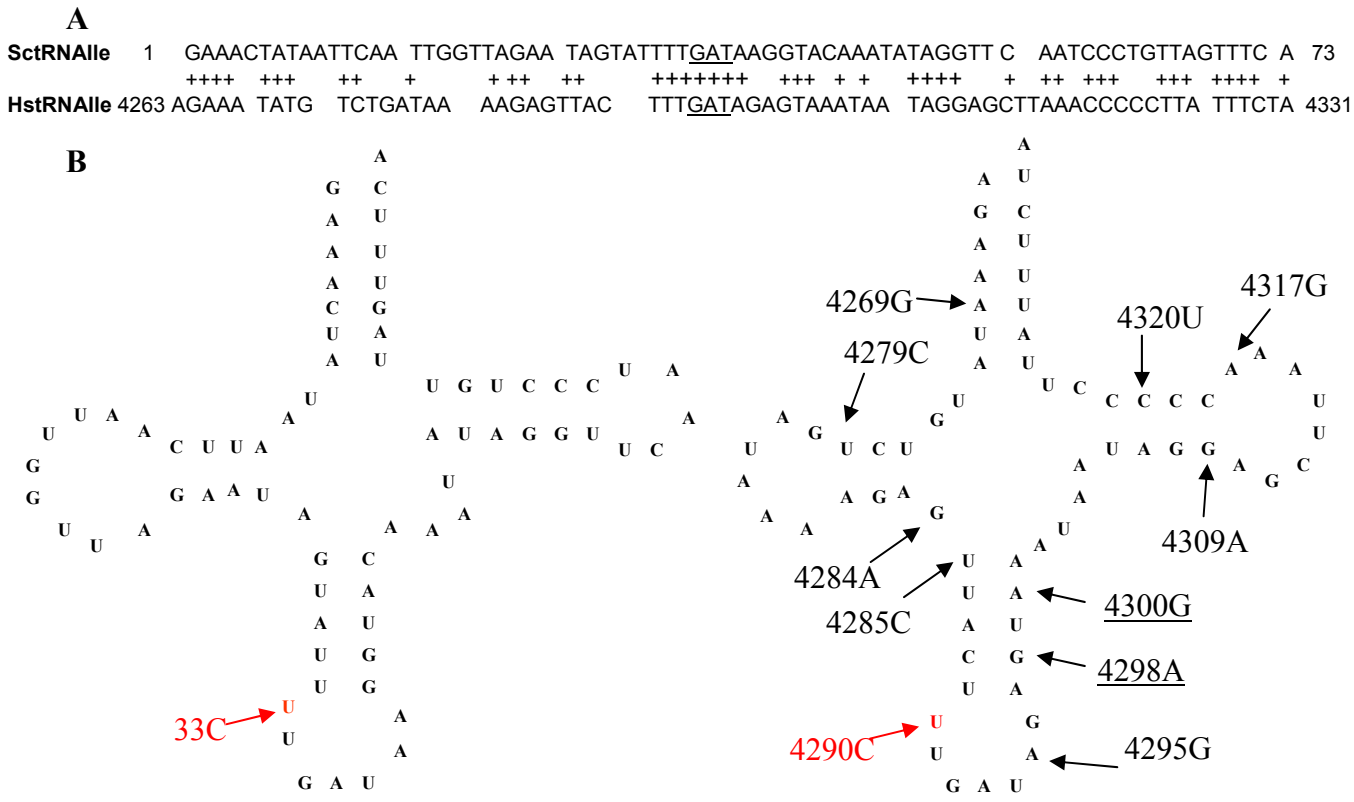


Fig. 16 Comparison of yeast and human tRNAIle sequences and secondary structures. Panel A shows the alignment between the tRNA gene sequences. Plus signs indicate the conserved positions between the two genes. The anticodon is underlined. Panel B represents the yeast (left) and human (right) cloverleaf structure. In the yeast molecule are indicated the mutations studied in this thesis. In the human molecule the arrows indicate the positions correlated with pathologies (www.mitomap.org). The underlined positions indicate the mutations that have been confirmed as pathological. The red colour indicates the position analysed in this work.

```
69468                    CCG GAACCCCGAA AGGAGAATAA TATAAAATAT
69501  TATAATTATT TATATATTAA TTATTAATTA TTTATTATTT ATTATATAAA
69551  AAGTATATAA TTTTATATTT TAATATAGGG TTAATTAATT AATTATTAAT
69601  TTTTTATAAT AAGATAATAA TATATTAAAA ACTTATTATA AATTTATAAA
69651  ATAATATTTA TTTACTTTGA TATTATTTTT AATCTTTCAT TAATATATAT
69701  TTTATTATAA GTAATAATAT AGTTTAATTT AATTAATATA AATAAATTAC
69751  ATAAGAATAA TATTATAATA ATATTATATA TTATATAAAG AAATAATAAT
69801  TTATATTTTT ATTTTTTTTA TAAATAATAT AAATATAAAT ATAATGGGGT   ALA
69851  TATAGTTAAA TTTGGTAGAA CGACTGCGTT GCATGCATTT AATATGAGTT
69901  CAAGTCTCAT TAACTCCAAT AATTATATTA TATAATATAT ATATTAATAA
69951  ATTATATATA TATATATATA TATAAATATT AAATAAATAT TATATTAATA
70001  AATAATATAA ATTATCTAAT CGAAGGAGAT ATTTATAATA TAATATAAAT
70051  ATTTTAATAA ATTAATAAAT ATTATATTAA TAAATAATTA ATAAATATAT
70101  AAATTATAAT AAATTTTAAT ATTATTATAT AAATTAATTA AATATAATAA
70151  TTAATGAAAT AGAAACTATA ATTCAATTGG TTAGAATAGT ATTTTGATAA   ILE
70201  GGTACAAATA TAGGTTCAAT CCCTGTTAGT TTCATATTAT ATATCATTAA
70251  TATATAAAAT ATAAATATAT ATATTATAAT AATAATAATA ATAAATATAA
70301  ATATAATTAT ATATATATAT ATATATAAAT AAATAATTAT TTAATTTATA
70351  ATAAATATAT ATAGTTCCCG CGAAGCGGGA ACCCCATAAG GAGTTTTATT
70401  ATTAATTATA TTTAATAAAT ATTAATTATT AATTTTATAT TTATAAATAA
70451  ATTTATTACT CCTTCTTAAT TAAGAATAAA AAGGGATGCG GTTCCCATGG
70501  GGTCCCGCAC TCCTTCGGGG TCCGCCCCCT CCCCTGCGGG AGGGGAGCGG
70551  ACTATTTTAT TAAAAATATT ATAATTAAAT AATAATATAA ATAATTTATA
70601  ATATAATAAT ATATAC
```

Fig. 17 Region of mitochondrial DNA containing tRNAIle gene cloned in the pJM2 biolistic vector. The tRNA gene of interest is indicated in red, while another tRNA gene is indicated in green.

3.2 Characterization of the mutants

For the LysG38A mutant it was possible to verify the presence of the substitution by restriction analysis. In fact this mutation introduces in the tRNALys gene one extra restriction site for the DraI enzyme. The mtDNA was extracted from the hypothetical recombinant colonies. After the PCR, using Leu^{+} and $Arg1^{-}$ primers, the recovered 1200 bp fragment

containing the tRNALys gene was checked for the presence of the DraI restriction site (Fig. 18).

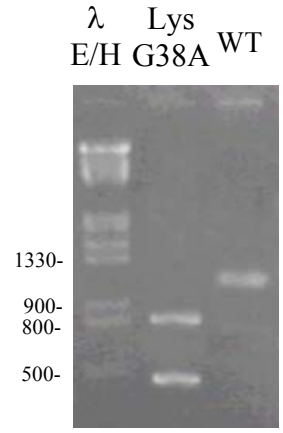


Fig. 18 DraI restriction of the PCR fragments amplified from the LysG38A mutant and the wild type.

LysG38A mutant was analysed for its growth phenotype on different non fermentable media. To perform this analysis serial cell dilutions from 10^{-3} to 10^{-7} were plated on 3 % glycerol, 2 % lactate and 6 % ethanol media at 28°C and 37°C (Fig. 19). At the permissive temperature of 28°C the mutant showed a slower growth on ethanol, lactate and glycerol containing media compared to the wild-type cells, while its growth was absent at 37°C.

From the preliminary screening for the selection of LysG38C and IleT33A mutants, colonies with slower growth on glycerol containing media at 28°C and absence of growth at 37°C were isolated. MtDNA was extracted from these cells and amplified by PCR using Leu^{+}-$Arg1^{-}$ oligonucleotides for LysG38C and $Arg2^{+}$-$Tyr2^{-}$ for IleT33A. The sequence of the amplified fragments to confirm the presence of the mutations is in progress.

In order to analyse the presence of tRNALys, Northern blot experiments were performed for the LysG38A mutant. Total RNAs from mutant and wild-type purified mitochondria were extracted under acidic conditions (pH 5); this condition is able to maintain the acylation status of the tRNA molecules.

The samples (10 μg for each RNA extract) were fractionated in 6 % polyacrylamide-8M urea gel (18 hours at 380V). The RNAs were then transferred to nitrocellulose filters and hybridised with the 5'-end labelled LYS probe and GLN probe as control (Fig. 20). Fig. 20 panel A shows that the mutated transcript was present in normal quantity and its migration was similar to that of the wild-type transcript.

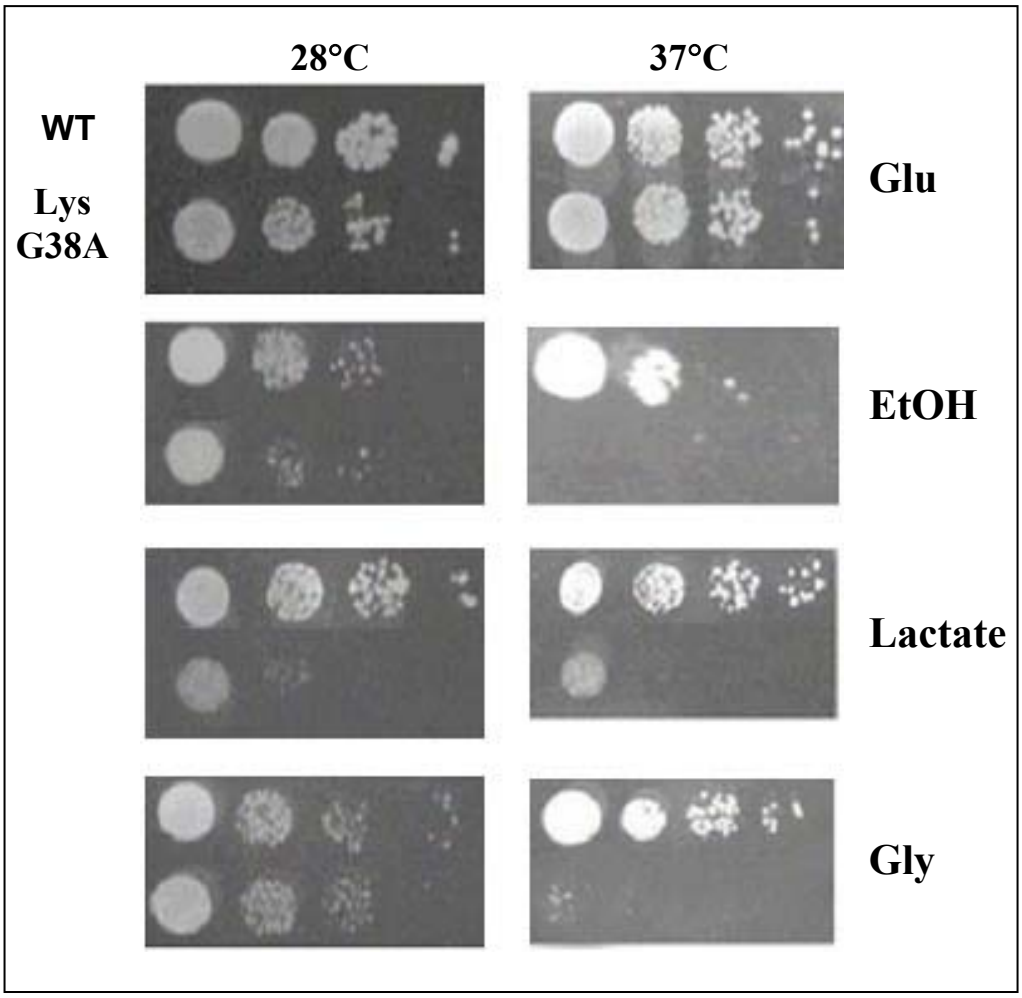


Fig. 19 Growth comparison of serial cell dilutions of wild type and LysG38A mutant at 28°C and 37°C on different carbon sources, 2% glucose, 3% glycerol, 2% lactate and 6% ethanol.

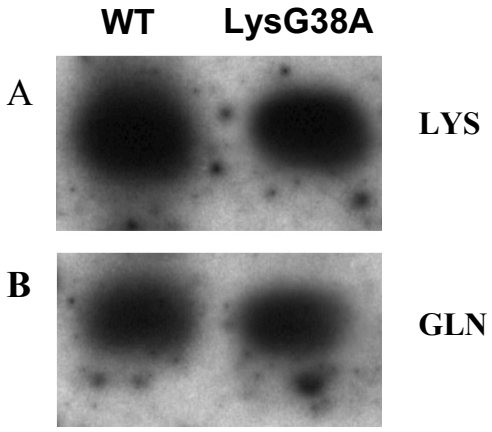


Fig. 20 Northern blot analysis of mitochondrial transcripts. Total RNAs extracted from purified mitochondria of wild type and LysG38A cells, were fractionated on polyacrylamide-urea gel. The mitochondrial RNAs were extracted under acidic conditions from cells grown at 28°C (A). Hybridization was with the 5'-end labelled LYS probe (panel A) or GLN probe as control (panel B).

3.3 Characterization of other mutants in the anticodon stem

3.3.1 x14.25 mutant

Berlani *et al.* have isolated a large number of mitochondrial mutants by $MnCl_2$ mutagenesis treatment, showing a defective growth phenotype on glycerol containing media (Berlani *et al.* 1980).
Experiments of complementation using different rho- mutants retaining specific sections of the mitochondrial DNA allowed Berlani and his collaborators to locate some of their mutants in the tRNA region.
The mutant denominated x14.25 showed absence of growth on glycerol containing media. The complementation experiments located the mutation in a region containing the mitochondrial tRNAThr1 gene (Macino *et al.* 1979). A region of 600 bp containing this gene was amplified by PCR using oligonucleotides 5'Thr1$^+$ and Val$^-$ and sequenced using the 5'Thr1$^+$ oligonucleotide. The tRNAThr1 gene sequence showed a C to T transition in position 40 and an A to T transversion at position 73. Fig. 21 shows the secondary structure of the tRNAThr1 in which the mutations are indicated.

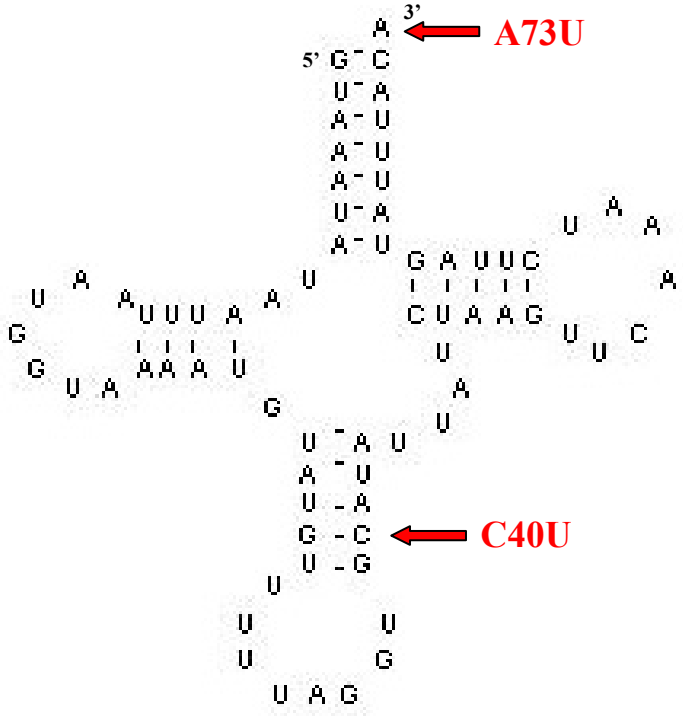


Fig. 21 tRNAThr1 secondary structure. Red arrows indicate the mutated positions in x14.25 mutant.

In order to verify the presence of tRNAThr1 transcript, total RNAs were extracted from the purified mitochondria of this mutant and from the wild type and analysed by Northern blot experiments. As previously described, the RNAs were extracted under acidic conditions from cells grown at 28°C and 37°C. The filter was hybridised with the 5'-end labelled THR1 probe.

To visualize the transcript it was necessary to load three-fold the usual quantity, i.e. 30 μg, of RNAs. Fig. 22 panel A shows that even if the mutated transcript was present in the x14.25 extracts the amount of this tRNA was much lower in the mutant cells compared to the wild-type ones. This amount is probably not sufficient to support a normal mitochondrial protein synthesis. An aliquot of RNA extracts was then treated with Tris base to hydrolyse the ester linkage between the tRNA and the cognate amino acid (deacylated samples). After the deacylation treatment it was possible to visualize only a band migrating faster compared to the acylated samples indicating that the few molecules of tRNA were acylated. Moreover, Fig. 22 shows no difference in the electrophoretic migration between the wild-type and the mutant samples. Fig. 22 panel B shows the hybridisation with ASP probe as a control of the quality and quantity of the samples; this control demonstrates that the effect of the mutation is specific for tRNAThr1.
As the mitochondrial DNA has two genes for threonine tRNA the frequency of occurrence of the Thr1 codon (CTA) was analysed. A Count Codon program available on line at the www.kazusa.or.jp web site was used. From this analysis it appears that the codons recognized by Thr1 have a frequency of 28.3 (per thousand); this value indicates that these codons are not less represented when compared to others.

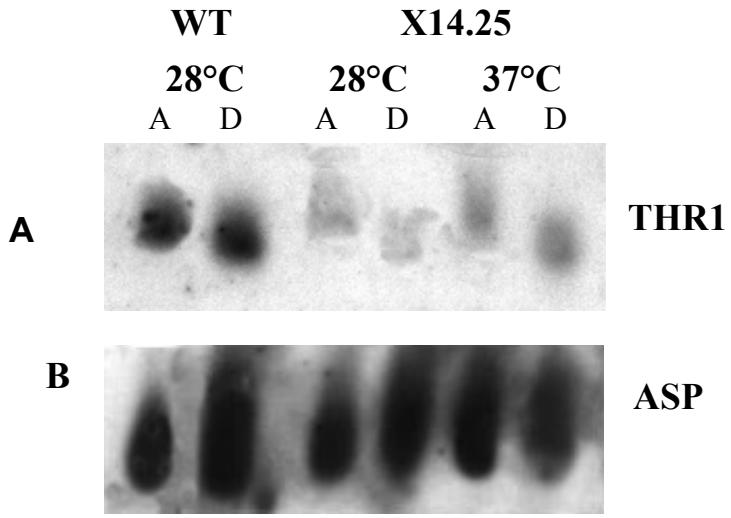


Fig. 22 Northern blot analysis of mitochondrial transcripts. Total RNAs extracted from purified mitochondria, isolated from wild type and x14.25 mutant, were fractionated on polyacrylamide-urea gel. The mitochondrial RNAs were extracted under acidic conditions from cells grown at 28°C and 37°C (A), and then treated to deacylate the tRNA (D). Hybridization was with the 5'-end labelled THR1 probe (panel A) and ASP probe as control (panel B).

3.3.2 U42C mutant

Another mutant isolated by Berlani *et al.* showed a weakly phenotype on glycerol media, growing slowly compared to the wild type at both temperatures of 28°C and 37°C.

The mtDNA region found positive by complementation experiments by Berlani was sequenced and the mutation was localized in the mitochondrial tRNAIle gene amplified by PCR using $Arg2^{+}$-$Tyr2^{-}$ oligonucleotides. The sequence revealed a T to C transition at position 42 (Fig. 23).

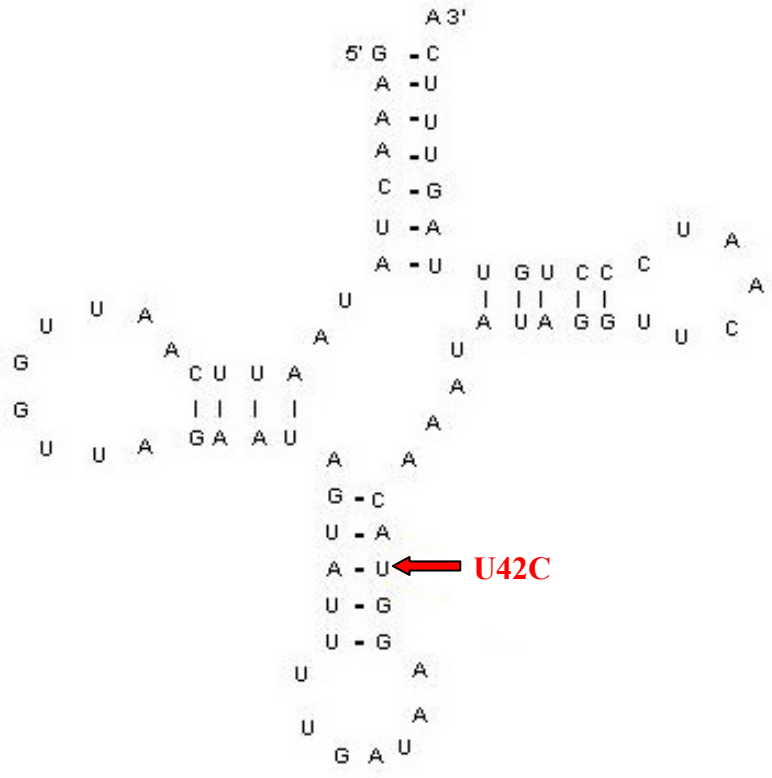


Fig. 23 tRNAIle secondary structure. Red arrow indicates the mutated position in U42C mutant.

Also for this mutant the presence and the acylation state of the tRNAIle transcript were analysed. The RNAs were extracted maintaining the acylated conditions from purified mitochondria of cells grown at 28°C and 37°C, and treated for deacylation. Fig. 24 shows that the tRNAIle was present and correctly acylated as compared to the wild type. Moreover, the electrophoretic mobility of mature U42C tRNAIle was similar to that of wild-type molecule, indicating that the mutated tRNAs do not undergo major structural distorsions in these conditions. In wild type and in U42C mutant, two bands were present corresponding to the acylated and deacylated forms of tRNAIle. The presence of two bands in the acylated conditions in both mutant and wild-type transcripts was already observed in other tRNA transcript analyses.

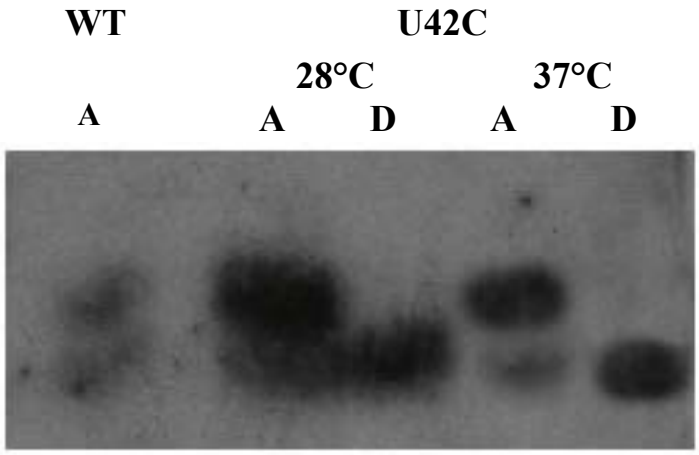


Fig. 24 Northern blot analysis of mitochondrial transcripts. Total RNAs extracted from purified mitochondria, isolated from the wild type and IleU42C mutant, were fractionated on polyacrylamide-urea gel. The mitochondrial RNAs were extracted under acidic conditions from cells grown at 28°C and 37°C (A). Samples were then treated to deacylated the tRNA (D). Hybridization was with the 5'-end labelled ILE probe.

3.4 Phenotype correction

3.4.1 Suppression by the mitochondrial-tRNA synthetases

It was demonstrated that a multi-copy plasmid bearing the nuclear gene MSD1, encoding for the mitochondrial aspartyl-tRNA synthetase, was able to suppress the growth deficient phenotype of a tRNAAsp mutant; the mutant has a substitution from C to T in position 62 of the T stem (Rinaldi *et al.* 1997). Similar results were also obtained with a mutant bearing a substitution in the acceptor stem of tRNAPhe; this mutant defective phenotype was suppressed by its specific synthetase (Francisci *et al.* 1998).
On the basis of these experiments, the effect of an over expression of the mitochondrial Leucyl-tRNA synthetase, encoded by NAM2, on the biolistic tRNALeu mutants (C26T, T69C and A14G) (Feuermann *et al.* 2003) was investigated. The NAM2 gene was cloned into pFL39, a centromeric vector, under the inducible GAL promoter (pCNAM2)(Herbert *et al.* 1988), and into pFL44 a multi-copy vector (pENAM2) (kindly provided by Prof. Claude Jacque).
These two vectors were used to transform tRNALeu mutants that showed a drastic phenotype on glycerol, as described in the introduction (par. 1.10.1). The transformation was performed using the LiCl protocol described in material and methods (par. 2.17). The transformants with the centromeric vector were plated on 3 % glycerol and 3 % glycerol/0.4 % galactose media. This percentage of galactose was sufficient to induce the GAL promoter but not to support the growth of the mutant strains (Fig. 25). LeuT69C and A14G mutants were suppressed by the presence of the centromeric vector without the galactose induction; whereas LeuC26T mutant was able to grow only when the GAL promoter was induced by the galactose containing media or when transformed with the multi-copy plasmid (pENAM2).

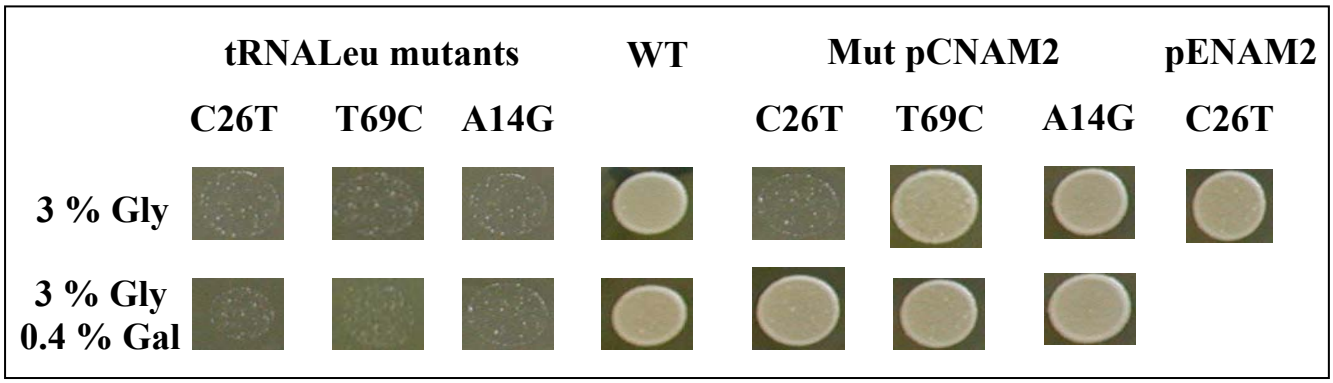


Fig. 25 Suppression of tRNALeu mutants C26T, T69C and A14G by NAM2 gene cloned into the centromeric vector pCNAM2 (under the Galactose inducible promoter) or into the multi-copy pENAM2.
The transformed strains were plated on glycerol and glycerol/galactose containing media.

Previous Northern Blot experiments with LeuC26T mutant showed that the tRNA transcript was not detectable because this mutant has a rapid loss of mtDNA (Feuermann *et al.* 2003). In order to analyse the effect of the Leucyl-tRNA synthetases on the transcripts of this mutant, mitochondrial total RNAs were extracted from LeuC26T and LeuC26TpNAM2 after an over-night growth on glucose and after 8, 24 and 48 hours of induction by galactose. The RNAs were then fractionated on a polyacrylamide gel and hybridised with LEU probe and VAL probe as control. Fig. 26 shows that the tRNALeu in the mutant was detectable only after 24 hours, and the signal was more evident after 48 hours. Furthermore, the mutated tRNALeu migrated more slowly than the wild type. In these electrophoretic conditions molecules are separated depending not only on their length but also on their secondary structure.

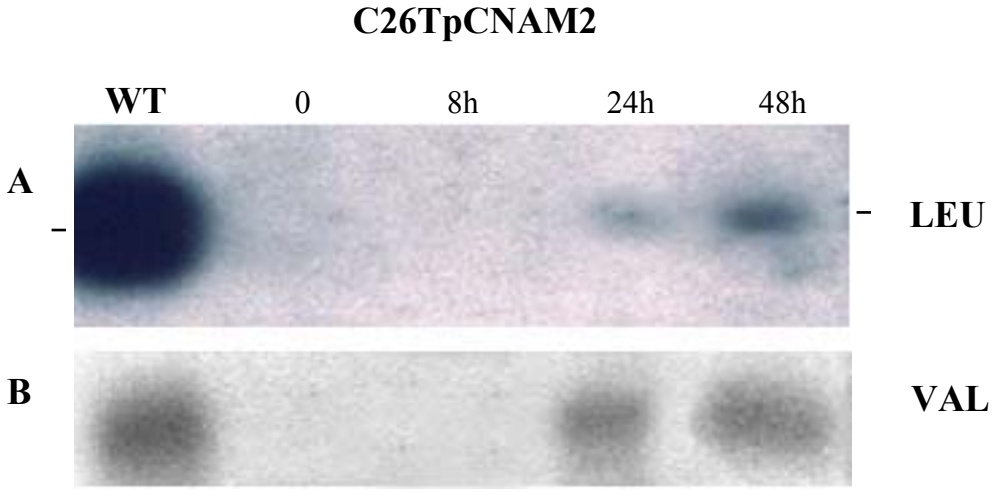


Fig. 26 Northern blot analysis of tRNALeu C26T mutant transformed with the vector pCNAM2. RNAs were extracted from wild-type and mutant strains after glucose growth. The transformed mutant was then shifted to galactose containing media and RNAs were extracted after 8, 24 and 48 hours. Hybridization was with the 5'-end labelled LEU probe (panel A) and VAL probe (panel B) as control.

The capability to rescue the tRNA mutant defective phenotype by the mitochondrial-tRNA synthetases was tested also with the biolistic mutant LysG38A. The nuclear gene (MSK1) coding for the mitochondrial Lysine-tRNA synthetase cloned into a centromeric plasmid pRS416 was kindly provided by Prof. I. Tarassov Laboratory.
The plasmid containing MSK1 gene and the nuclear marker URA3 was used to transform the LysG38A mutant. Transformants, selected on minimum media without uracile, were tested for their ability to grow on glycerol at 28°C and 37°C. The growth efficiency of transformants was compared to that of the wild-type and mutant strains using serial cell dilutions. Fig. 27 shows that few more copies of MSK1 gene were sufficient not only to restore a wild-type phenotype on glycerol containing media at 37°C but also to improve the growth rate compared to the wild type.

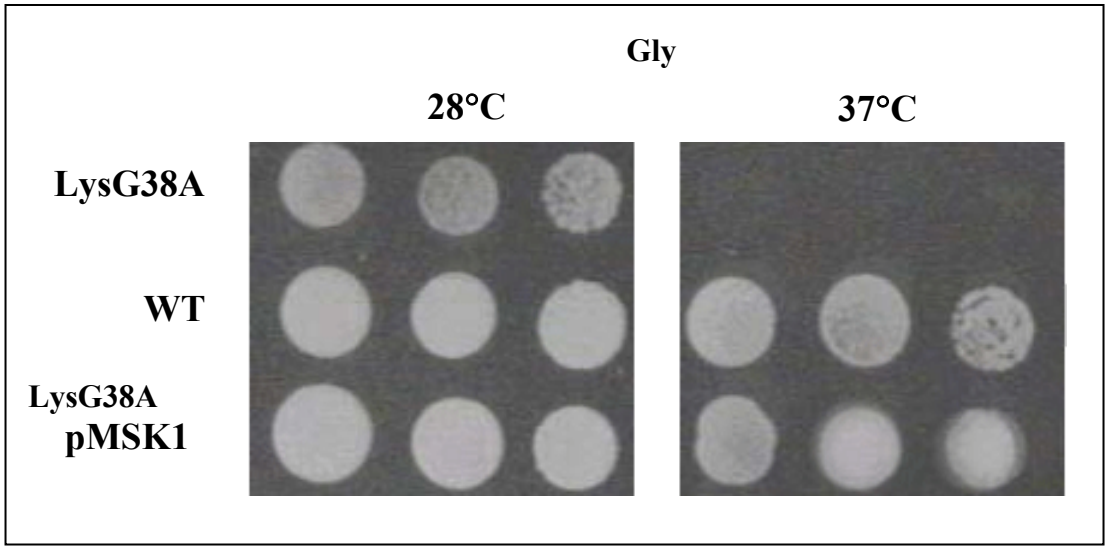


Fig. 27 Growth comparison of mutant LysG38A, wild type and LysG38A transformed with a vector containing the Lysyl-tRNA synthetase gene (MSK1). The three different strains were plated on glycerol containing media and compared using serial cell dilutions.

3.4.2 Suppression of mutants by EF-Tu

Previous works (Rinaldi *et al.* 1997, Francisci *et al.* 1998, Feuermann *et al.* 2003) reported that the mitochondrial elongation factor (EF-Tu) was able to suppress the defective growth phenotype of a large number of tRNA mutants.
The nuclear encoded TUF1 gene (for the EF-Tu) with its own promoter was recleaved by EcoRI from the multi-copy plasmid YEpLAC181TUF1 (here indicated as p181TUF1) containing the LEU2 yeast selective marker (Rinaldi *et al.* 1997). The fragment was subcloned into pFL61 multi-copy vector (containing the URA3 marker)(Bonneaud *et al.*1991). The PCR amplified fragment containing only the TUF1 gene (from p181TUF1) was also cloned in the pFL61 vector in the NotI restriction site (pFL61TUF1$_{NOT}$) (as described in

Materials and Methods par. 2.3). This construction permitted the expression of the TUF1 gene controlled by the PGK1 promoter, which is the phosphoglycerate kinase strong promoter.
LysG38A, x14.25, and Ts9 mutants were transformed with three different plasmids containing TUF1 and selected on minimum media for the presence of the plasmid. The ability of the transformed mutants to grow on glycerol containing media was tested at 28°C and 37°C.
Ts9 mutant derived from the mutagenesis of FF1210-6C (Elelj-Fridhi *et al.* 1991) and was previously characterized by Rohou to have a transition from G to A in position 30 in the anticodon stem of tRNAGly (Rohou *et al.* 2001). Its phenotype resulted thermo-sensitive on glycerol containing media.
The data reported in Table 2 show the results of the transformations. It is possible to observe that not all the mutants were suppressed by multi-copy plasmids containing the TUF1 gene. LysG38A and Ts9 mutants were suppressed by this factor in each of the cloned version. x14.25 mutant was not suppressed by any of the three TUF1containing plasmids. U42C mutant has a weak phenotype so it was not possible to tested its suppression.

Table 2 Analysed mutations of yeast mitochondrial tRNAs. Mutants are indicated by their mutation type and position. (+) indicates normal growth, (-) indicates absence of growth, and (+/-) indicates slow growth compared to the wild type.

Mutants	Localization of the mutation	Gly		Transformation	Suppression	
		28 °C	37°C		28°C	37°C
LysG38A	G38A tRNALys	+	-	pFLTUF	+	+
				pFLTUFnot	+	+
x14.25	C40T tRNAThr1	-	-	pFLTUF	-	-
				pFLTUFnot	-	-
				p181TUF	-	-
Ts9	G30A tRNAGly	+	-	p181TUF	+	+
				pFLTUF	+	+
				pFLTUFnot	+	+
U42C	T42C tRNAIle	+/-	+/-	/	/	

3.5 Nuclear backgrounds

3.5.1 Different nuclear backgrounds

It was interesting to observe that only the x14.25 defective phenotype was not rescued by the TUF1 containing plasmids. As this mutant derived from the mutagenesis of a different wild-type strain, it was decided to investigate the possibility that the nuclear background influences the expression of the mutated mitochondrial gene and the capability to be rescued by nuclear encoded proteins.
Three different wild-type strains were used to change the nuclear background:

- FF1210-6C, mat α, Δleu, ura1-2
- MCC123, mat a, Δleu, ade2, ura3, Kar1-1
- D273-10B/A1, mat α, Δleu, met, ura3 (Berlani *et al.* 1980)

These wild-type strains were treated with ethidium bromide to obtain rho° cells.
The nuclear mutation denominated Kar1-1 offers the advantage of avoiding nuclear fusion and therefore recombination between nuclei (Kurihara *et al.* 2004). Moreover, rho^+ cells with the ade2 mutation can be distinguished for their red colour, due to the accumulation of a red pigment; however, when mitochondria become dysfunctional these cells turn white (Kim *et al.* 2002).
Fig. 28 represents a cross between a mutant and a wild-type rho° strain, which is in this case ade2 and Kar1-1. After 12 hours on YPD medium, the cells were plated as single colonies in the same medium. By screening these plates, it is possible to distinguish different colonies for their size and colour (Fig. 29): big and white (10 % of diploids); medium and white (haploids ade2 unable to breathe or not defective in the adenine biosynthesis); medium and red (haploids retaining the ade2 mutation and able to breath); small and white (haploids rho°).
This strategy was used to change the nuclear context of x14.25, U41C and Ts9 mutants (the results are reported in Table 3). The D/x14.25, to indicate its origin from the mutagenesis of D273-10B/A1, was crossed with MCC123 wild-type strain (rho°) in order to isolate clones with the mutated tRNA and the nuclear background changed from D273-10B/A1 to MCC123 (denominated M/x14.25). To obtain the M/x14.25, medium red colonies were tested for their auxotrophies and growth phenotype on glycerol containing media at 28°C and 37°C. The M/x14.25 was then crossed with FF1210-6C (rho°) and medium white colonies were isolated and checked to obtain the F/x14.25. Originally unable to grow on glycerol containing media, this mutant showed a thermo-sensitive phenotype when the mutation was expressed in an MCC123 nuclear background and a wild-type phenotype when the mutation was expressed in a FF1210-6C nuclear background.
Similar experiments were conducted on the F/Ts9 mutant (derived from FF1210-6C), whose phenotype resulted thermo-sensitive on glycerol media. The M/Ts9 and D/Ts9 were tested on glycerol containing media; both mutants were unable to grow at 37°C or at 28°C.
The same experiment was repeated for the U42C mutant. Originally isolated from D273-10B/A1, this mutant showed a weak growth phenotype, growing slower when compared to the wild type. The M/U42C and F/U42C were both able to grow as wild type on glycerol media.
LysG38A mutant (mat a, ade2, ura3, Kar1-1), derived from the biolistic transformation of MCC123, was crossed with D273-10B/A1 and FF1210-6C wild-type strains (rho°) to obtain respectively the D/LysG38A and F/LysG38A mutants. The original phenotype of the mutant was temperature sensitive but resulted changed when associated to these different backgrounds. The worst phenotype resulted when the mutation was associated with a D273-10B/A1 nuclear context: this mutant was unable to grow on glycerol at 37°C or at 28°C, so that a DAPI staining was performed to confirm the presence of the mitochondrial DNA. After the DAPI (4',6-diamidino-2-phenylindole) treatment it is possible to visualize by fluorescence microscopy the DNA present inside the cell. The same mutation expressed into an FF1210-6C background showed no defective phenotype.
The experiment on the nuclear context suggested that the mitochondrial protein synthesis resulted less damaged when the tRNA mutations were associated to the nuclear context FF1210-6C; while the phenotype was more drastic when the tRNA mutations were associated to the nuclear context D273-10B/A1.

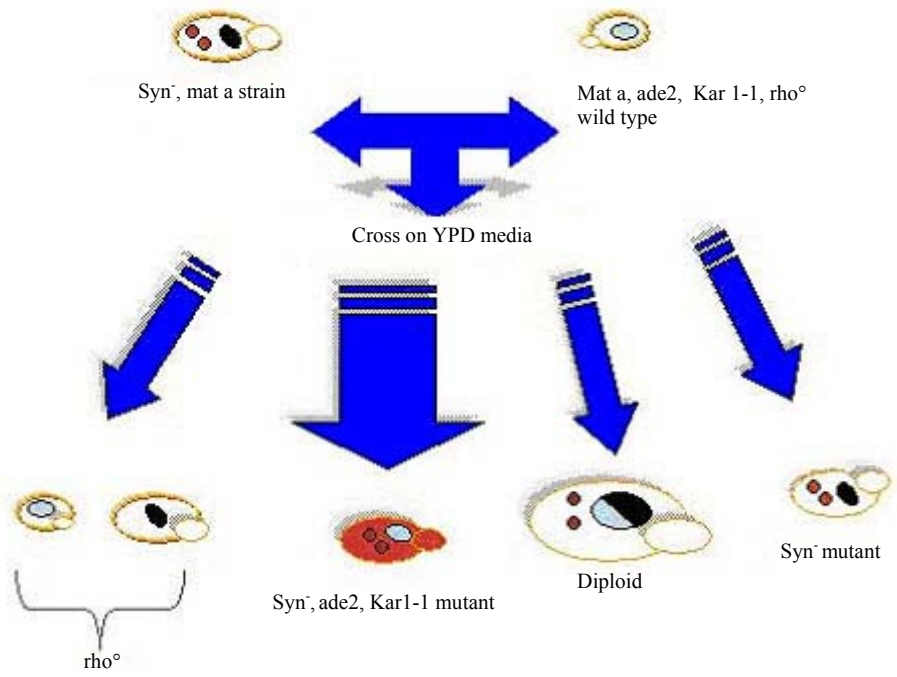


Fig. 28 Example of cross between a syn- mutant and a wild type to obtain the same syn- mutation in a new nuclear background.

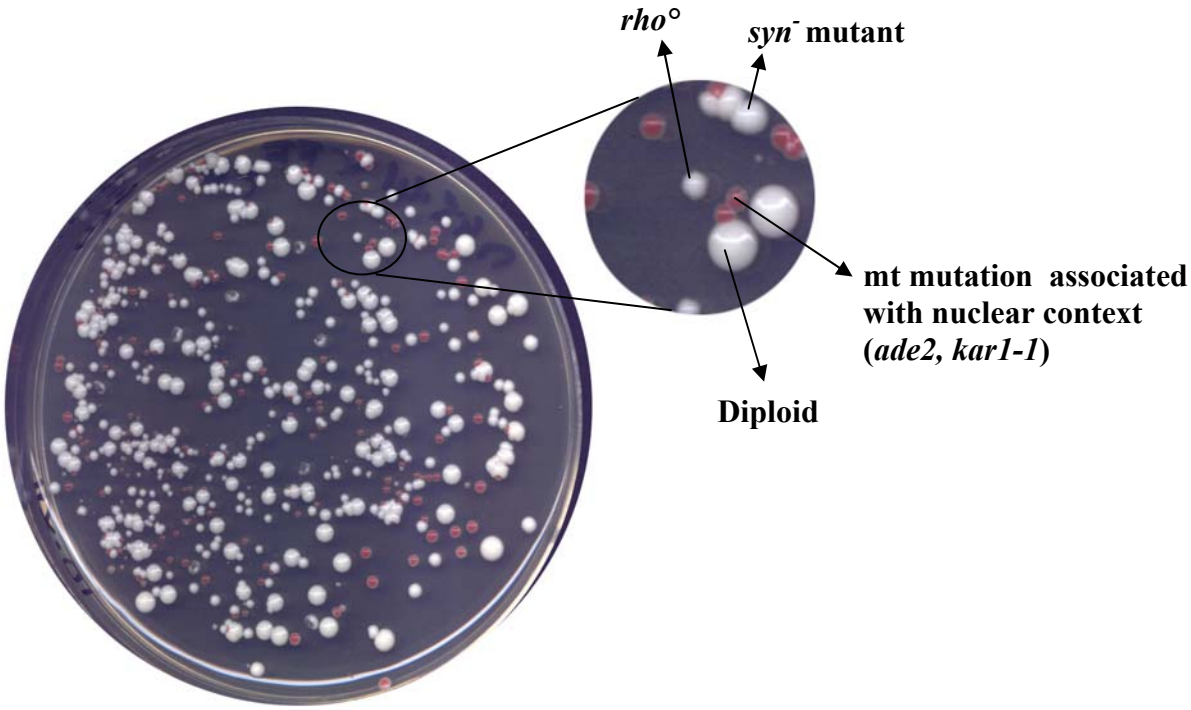


Fig. 29 Growth on glucose medium of colonies obtained by the cross of a syn- mutant with a wild type (rho°, ade2, kar1-1). It is possible to distinguish different colonies for their size and colour: big and white (10 % of diploids); medium and white (haploids not defective in the adenine biosynthesis); medium and red (haploids retaining the ade2 mutation and able to breath); small and white (haploids rho°).

All the mutants described above, in their changed nuclear contexts were transformed with the three different plasmids containing TUF1 gene. The results of these suppressions suggested that the rescue by the over expression of this factor is linked to the nuclear background in which the mutation was expressed (Table 3). From these experiments it is possible to conclude that phenotype suppressions not possible in the D273-10B/A1 nuclear context, had successful results in the MCC123 context. When the mutations were expressed in the F/ context almost no defective phenotype was observed. In the M/Ts9 strain the vector pFL61TUF1$_{NOT}$ was not able to rescue the defective phenotype. To test the possible effect of these plasmids, the three different wild-type strains were transformed with p181TUF1, pFL61TUF1 and pFL61TUF1$_{NOT}$ vectors (data not shown). From these transformations resulted that only the MCC123 with pFL61TUF1$_{NOT}$ had a slow growth on glycerol media at 37°C, indicating that the over expression of TUF1 gene under the PGK1 promoter may have toxic effects when associated with the M/ context.

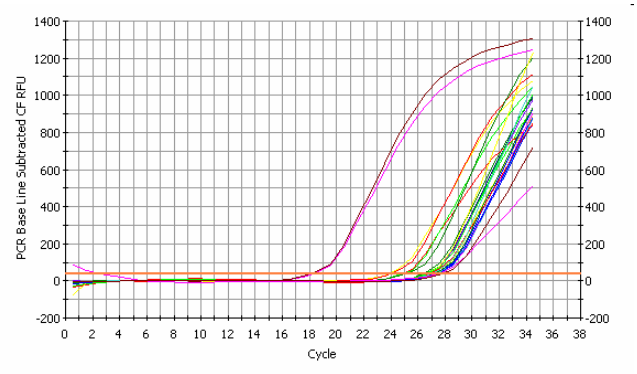
Table 3 Analysed mutations of yeast mitochondrial tRNAs.
Mutants were analysed in three different nuclear backgrounds:
F/ indicates that the mitochondrial mutation was expressed in the FF1210-6C context.
M/ indicates that the mitochondrial mutation was expressed in the MCC123 context.
D/ indicates that the mitochondrial mutation was expressed in the D273-10B/A1 context.

Mutants	Localization of the mutation	Gly		Transformations	Suppression	
		28 °C	37°C		28°C	37°C
D/LysG38A	G38A tRNA^{Lys}	-	-	pFLTUF	-	-
				pFLTUFnot	-	-
M/LysG38A	"	+	-	pFLTUF	+	+
				pFLTUFnot	+	+
F/LysG38A	"	+	+	/	/	
D/x14.25	C40T tRNA^{Thr1}	-	-	pFLTUF	-	-
				pFLTUFnot	-	-
				p181TUF	-	-
M/x14.25	"	+	-	pFLTUF	+	+
				pFLTUFnot	+	+
				p181TUF	+	+
F/x14.25	"	+	+	/	/	
D/Ts9	G30A tRNA^{Gly}	-	-	pFLTUF	-	-
				pFLTUFnot	-	-
				p181TUF	-	-
M/Ts9	"	-	-	pFLTUF	+	-
				pFLTUFnot	-	-
				p181TUF	+	-
F/Ts9	"	+	-	pFLTUF	+	+
				pFLTUFnot	+	+
				p181TUF	+	+
D/U42C	T42C tRNA^{Ile}	+/-	+/-	/	/	
M/U42C	"	+	+	/	/	
F/U42C	"	+	+	/	/	

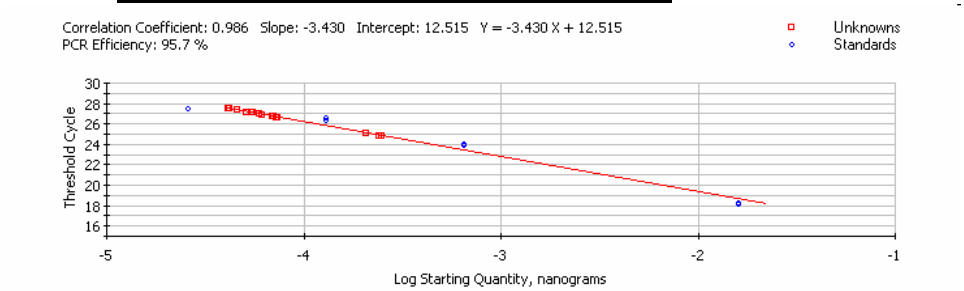
3.5.2 Level of EF-Tu

A large number of nuclear genes influence mitochondrial activities and in particular our experiments suggested that the level of EF-Tu may be crucial for the functionality of the tRNA mutated molecules.
It was decided to determine the absolute quantity of TUF1 gene expressed in different wild-type samples using a sensitive technique for DNA-RNA analysis such as the Real-time Polymerase Chain Reaction (RT-PCR).
Total RNAs were extracted from FF1210-6C (rho° and rho^{+}), MCC123 (rho^{+}) and D273-10B/A1 (rho° and rho^{+}) cells. To generate the copy DNA (cDNA), necessary for the experiment, 2 μg of RNA extracted from each strain were treated with DNase and used to perform a Reverse Transcriptase Reaction (as described in Materials and Methods).
In order to quantify the concentration of DNA target in these samples it was necessary to prepare standard samples of a known quantity and to design a standard curve. The YEpLAC181 vector containing TUF1 gene was carefully quantified and used to construct a standard curve, using serial dilutions starting from a $1.6\ 10^{-2}$ and ending at $2.56\ 10^{-5}$ng.
$TUF1^{+}$ and $TUF5^{-}$ oligonucleotides were chosen to perform the PCR reaction, in fact these primers amplified a region of 100 bp.
The samples were prepared with the SYBR green fluorophore, which is able to bind dsDNA. The fluorescence signal emitted by this fluorophore is read at 530 nm from the i-cycler apparatus and increases proportionally with the quantity dsDNA amplified. The SYBR green fluorophore does not discriminate among different dsDNA molecules so a further control using the Melt Curve is required. This curve is able to characterize the PCR product eventually revealing the presence of oligonucleotide dimers or PCR artifacts.
At the end of the reaction the software, provided by Biorad, analysed the fluorescence and provided a report with: a curve representing the threshold values (Fig. 30A), a Standard curve (Fig. 30B), and a table with all the threshold values (Fig. 30C).
The following increasing gradient of TUF1 transcript could be extrapolated: rho^{+} FF1210-6C, rho^{+} D273-10B/A1, rho° D273-10B/A1, rho^{+} MCC123, rho° FF1210-6C. Two graphics (Fig. 31) were drawn from the RT-PCR data.
These results suggest that the amount of TUF1 transcript is not very different among the three wild-type strains, but it was interesting to find that the major difference derived from the comparison between the rho° and rho^{+} strains. In particular the rho° FF1210-6C showed a level of TUF1 transcript five-fold higher than that of the rho^{+} strain suggesting the presence of a regulative mechanism in this wild type.

A **PCR Amp/Cycle Graph for SYBR-490**



B **Standard Curve Graph for SYBR-490**



C **Standard Curve Spreadsheet Data for SYBR-490** **Units: nanograms**

Type	Identifier	Ct	Log SQ	SQ	SQ Mean	SQ SD	Ct Mean	Ct SD
Standard	1	18.29	-1.796	1.60E-02	1.60E-02	2.11E-10	18.24	6.90E-02
Standard	1	18.19	-1.796	1.60E-02	1.60E-02	2.11E-10	18.24	6.90E-02
Standard	2	1,0243056	-3.194	6.40E-04	6.40E-04	1.31E-12	24.02.00	9.52E-02
Standard	2	24.08.00	-3.194	6.40E-04	6.40E-04	1.31E-12	24.02.00	9.52E-02
Standard	3	26.60	-3.893	1.28E-04	1.28E-04	1.52E-12	26.51.00	1.35E-01
Standard	3	26.41.00	-3.893	1.28E-04	1.28E-04	1.52E-12	26.51.00	1.35E-01
Standard	4	27.53.00	-4.592	2.56E-05	2.56E-05	3.00E-13	27.52.00	1.48E-02
Standard	4	27.50.00	-4.592	2.56E-05	2.56E-05	3.00E-13	27.52.00	1.48E-02
Unknown	MCC	26.73	-4.144	7.18E-05	6.57E-05	9.11E-06	26.87	2.16E-01
Unknown	MCC	26.77	-4.154	7.01E-05	6.57E-05	9.11E-06	26.87	2.16E-01
Unknown	MCC	27.12.00	-4.258	5.53E-05	6.57E-05	9.11E-06	26.87	2.16E-01
Unknown	FF rho°	24.94	-3.621	2.40E-04	2.31E-04	2.10E-05	25.00.00	1.39E-01
Unknown	FF rho°	24.90	-3.609	2.46E-04	2.31E-04	2.10E-05	25.00.00	1.39E-01
Unknown	FF rho°	25.15.00	-3.685	2.07E-04	2.31E-04	2.10E-05	25.00.00	1.39E-01
Unknown	FF rho+	27.41.00	-4.342	4.55E-05	4.59E-05	5.00E-06	27.40.00	1.62E-01
Unknown	FF rho+	27.24.00	-4.292	5.10E-05	4.59E-05	5.00E-06	27.40.00	1.62E-01
Unknown	FF rho+	27.56.00	-4.387	4.10E-05	4.59E-05	5.00E-06	27.40.00	1.62E-01
Unknown	D273 rho°	26.70	-4.135	7.32E-05	6.78E-05	6.08E-06	26.82	1.36E-01
Unknown	D273 rho°	26.97	-4.213	6.12E-05	6.78E-05	6.08E-06	26.82	1.36E-01
Unknown	D273 rho°	26.79	-4.162	6.88E-05	6.78E-05	6.08E-06	26.82	1.36E-01
Unknown	D273 rho+	27.16.00	-4.269	5.38E-05	5.17E-05	9.15E-06	27.24.00	2.75E-01
Unknown	D273 rho+	27.01.00	-4.225	5.95E-05	5.17E-05	9.15E-06	27.24.00	2.75E-01
Unknown	D273 rho+	27.54.00	-4.381	4.16E-05	5.17E-05	9.15E-06	27.24.00	2.75E-01

Fig. 30 Reports from the RT-PCR analysis.

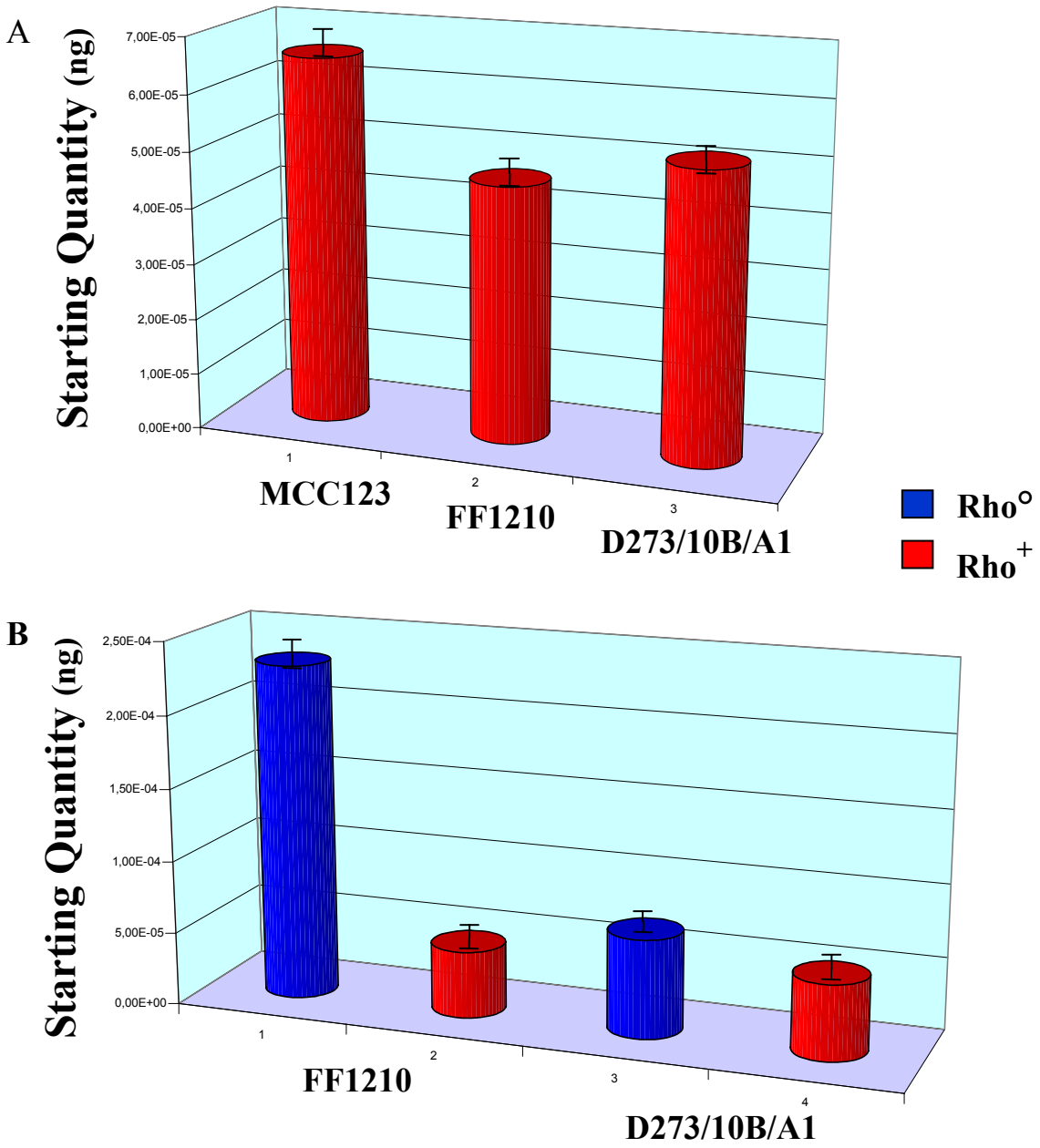


Fig. 31 Graphic representation of TUF1 transcript quantification resulted from RT-PCR using cDNA from different wild-type strains. Panel A shows the comparison among the three different rho$^+$ strains. Panel B shows the comparison between rho° and rho$^+$ strains.

Discussion

4.1 Characterization of different mutants in the anticodon stem

In this thesis the effect of point mutations in three mitochondrial tRNA genes has been analysed. As no mutants obtained by random mutagenesis reproduce in yeast mutations correlated in humans with neurodegenerative pathologies, the biolistic transformation was used to introduce specific point mutations.
From the biolistic transformation and subsequent screening two mutants in position 38 of the mitochondrial tRNALys were isolated, in particular a transition from G to A (correlated in humans with the pathologies by Houshmand) and a transversion from G to C. Both these substitutions cause growth defective phenotype on glycerol containing media, which indicates problems in the mitochondrial protein synthesis. LysG38A mutant showed a temperature sensitive growth phenotype (no growth at 37°C) while the other mutant LysG38C showed a slow growth even at 28°C.
The nucleotide G in position 38 is conserved in almost all the eukaryotic tRNALys, but the base-pairing is not conserved. In yeast the G in position 38 binds the U in position 30 of the anticodon stem; this bond is less strong than the canonical Watson-Crick base-pair G-C found in the human tRNALys. Consequently, the G to A transition in yeast introduces a strong bond in the anticodon stem, while in humans the mutation has the opposite effect (the loss of a GC strong base-pair). It would be interesting to introduce a C 30 mutation to obtain in yeast the same base-pair present in the human molecule.
Preliminary screening results on LysG38C mutant suggest that the G to C substitution causes in yeast tRNALys a worst phenotype comparing to the LysG38A mutant, probably due to the loss of the base-pairing.
In the mitochondrial tRNAIle only one biolistic mutant, with a T to A transversion in position 33, was isolated. This mutant showed a slow growth phenotype only at 28°C on glycerol containing media, confirming that the substitution of this nucleotide causes effects in yeast as well as in humans. The anticodon loop of this tRNA is highly conserved from humans to yeast and it would be interesting to verify the specificity of the nucleotide substitution characterizing the other two mutants, IleT33G and IleT33C, not yet obtained.
The Northern blot experiment with LysG38A mitochondrial RNA extracts showed that the signal corresponding to the tRNA transcript was present and had the same electrophoretic mobility of the wild-type molecule, indicating no structural defect of the molecule of tRNA at least at 28°C; further investigation at 37°C will complete the analysis of this mutant transcript. As LysG38A mutant showed growth deficiencies not only on glycerol but also on other non fermentable media (lactate and ethanol were tested), it is possible to hypothesize that this base of the anticodon stem is important for the interactions of this tRNA molecule with its partners during the protein synthesis.
Other analysed mutants, not related to pathological substitutions in humans but located in the anticodon stem, were obtained by random mutagenesis. Even if the mutations were located in different tRNA genes, it is possible to speculate on the relevance of the mutated positions for the efficient folding of the molecules and for their interactions with other molecules.
x14.25 mutant has two substitutions one localized in position 40 and the other in position 73 of the tRNA. This mutant showed absence of growth on glycerol media, indicating that the mutations cause strong defect in the mitochondrial protein synthesis. The mutation in position 40 causes the loss of a canonical Watson-Crick (C-G) base-pair probably destabilizing the anticodon stem. The mutation in position 73 of the

acceptor stem is not involved in secondary interactions within the molecule, but it is known to be an identity element for the aminoacylation reaction in some tRNAs.
The Northern blot analysis performed with mitochondrial RNAs extracted from x14.25 revealed low levels of the transcript compared to the wild type. To visualize the mutant signals was necessary to over load the samples three-fold more than the usual quantity. This experiment revealed no differences in the secondary structures nor in the aminoacylation between the tRNAThr1 extracted from the mutant and the wild type. Therefore this experiment suggested that positions 40 and 73 are not identity elements for this tRNA. Since this mutant is not able to growth on glycerol media, the low level of the tRNAThr1 transcript is probably not sufficient to support the mitochondrial protein synthesis. It was observed that mutated tRNAThr1 could not be compensated by the tRNAThr2; these two tRNAs recognize different codons. From the analysis of the frequency of occurrence resulted that codons recognized by tRNAThr1 are not less represented compared to others.
The results obtained with x14.25 mutant are in agreement with those obtained for the Ts9 mutant (Rohou *et al.* 2000). This mutant has the substitution G30A in the tRNAGly; this position is involved in the same secondary interaction G30-C40 as x14.25 mutant. This mutant showed a thermo-sensitive phenotype on glycerol media and, similarly to x14.25 mutant, showed low level of transcript. It is therefore possible to hypothesize that position 40, in agreement with the results observed for Ts9 mutant, is important for the steady state level of the transcript probably affecting the stability of the tRNA molecule, at least for tRNAThr1 and Gly.
U42C mutant has a substitution which causes the loss of a canonical Watson-Crick (A-T) base-pair of the anticodon stem of tRNAIle. Since structure or aminoacylation defects were not observed for the mutated tRNAIle by Northern blot analysis, position 42 seems not to be an identity nucleotide for the yeast mitochondrial tRNAIle. Moreover, two bands were present in the mitochondrial extract from the wild-type and mutant cells corresponding to the acylated and deacylated sample of tRNAIle. The presence of two bands was previously observed for tRNAHis, in fact the cell may maintain both the acylated and the deacylated molecules for regulative mechanisms (Hinnebusch and Natarajan 2002). Due to the presence of the mutation, U42C mutant maintains only two strong base-pairs in the anticodon stem of tRNAIle; therefore, the respiratory defect could be the consequence of an alterated structure of the stem. In fact from database analyses it has been noted that tRNAs have usually five bonds in the anticodon stem; at least four base-pairs are, usually, strong bonds (Watson-Crick base-pairs). In only two tRNAs (one mitochondrial and one cytoplasmic tRNA) were observed three Watson-Crick base-pairs.
Summarizing the data on the mutants of the anticodon stem reported in this thesis, it is possible to observe that: the mutations here investigated do not cause problems in the structure and aminoacylation efficiency detectable by Northern blot experiments; the bond involving positions 30 and 40 (respectively in Ts9 and x14.25) is important for the steady state level of the tRNAs; mutations in LysG38A and U42C mutants, showing no defects detectable by Northern blot experiments, probably cause less efficiency in the interactions of the tRNA molecules with their partners.
Further modeling studies, in collaboration with the laboratory of Prof. A. Tramontano, permitted the determination of some important structural features of the mitochondrial biolistic tRNALeu mutants, in position A14G, C26T and T69C, previously characterized (Feuermann *et al.* 2003). In particular, an adenine in position 14 is conserved in almost all the tRNAs and it has a conserved tertiary interaction with the

nucleotide in position 8. This interaction may be essential for the correct folding of the molecule. Position 26 is involved in a conserved interaction with nucleotide in position 10. The substitution C26T causes the loss of the hydrogen bond between this position and the guanine at position 10 resulting in a destabilization of the secondary structure and consequently of the tertiary structure. The nucleotide in position 69 usually has a conserved interaction with D loop, but in yeast and humans this region has a different pattern; it is, therefore, difficult to determine a general mechanism which may explain this mutant phenotype. Recently, in humans, a model has been proposed to explain the effects of the A3243G mutation (corresponding to the yeast A14G)(Chomyn *et al.* 2000). This mutation alters the tertiary structure of the tRNA so that the molecule is deficient for the methylation of the G at nucleotide 3239 and for the modification of the uridine at the first position of the anticodon. As a consequence, tRNALeu(UUR) is less stable and less efficiently charged by its specific synthetase. The reduced ratio between charged and uncharged tRNA affects the association of mRNA to the ribosome. This will reduce the protein synthesis and exacerbate the phenotype.

4.2 Suppression by mitochondrial-tRNA synthetases and EF-Tu

It was demonstrated that the nuclear genes MSD1 and MSF1, encoding respectively for the mitochondrial Aspartyl-tRNA synthetase and Phenylalanyl-tRNA synthetase, can be utilized to suppress the defective phenotype of strains mutated in the mitochondrial tRNAAsp and tRNAPhe (Rinaldi *et al.* 1997, Francisci *et al.* 1998).
Mutants in the mitochondrial tRNALeu showed no growth on glycerol containing media. In this thesis the defective growth phenotype of these mutants was rescued by the over expression of NAM2 gene, encoding for the mitochondrial Leucyl-tRNA synthetase (LeuRS). This enzyme has an important role in the mitochondria, in fact it has an aminoacyl-tRNA synthetase activity and also a maturase activity, required for the correct splicing of cytochrome b gene. The LeuRS is a class I synthetase (Ibba and Soll 2000), the main characteristic of this class is the capability of the active site to bind the acceptor helix of tRNA on the minor groove site.
In this thesis has been reported that the A14G and T69C mutants were able to grow on glycerol when transformed with the centromeric version of the vector containing the NAM2 gene (pCNAM), while C26T mutant was suppressed only when the NAM2 gene was over expressed by the induction of the GAL promoter in the centromeric vector or by the multi-copy plasmid (pENAM2). The positive effect on the tRNA transcript was verified by the Northern blot experiment. Previous experiments reported that the C26T mutation causes the rapid loss of mtDNA. It was, therefore, neither possible to visualize the tRNALeu transcript nor other transcripts in this mutant. The tRNALeu was visualized only by Northern blot experiments with RNAs extracted from C26T cells transformed with a multi-copy plasmid bearing the TUF1 gene; a similar result was obtained, in this thesis, when the RNAs were extracted from the C26T transformed with pCNAM2. The tRNALeu transcript was detectable by the Northern blot experiment only after 24 hours of galactose induction. The electrophoretic migration of the mutated tRNA compared to the wild-type molecule confirmed that the C26T mutation causes alteration in the structure of the molecule. The conditional expression of NAM2 gene, due to the GAL promoter, offers the possibility to mimic, in some way, the effect of the heteroplasmy present in the human cells. By allowing the presence of functional and dysfunctional tRNA molecules in the yeast cells, it is, therefore, possible to produce intermediate effects. Moreover, these experiments confirm that the functionality of the

molecule is rescued not only by the over expression of the EF-Tu (as reported by Feuermann *et al.* 2003) but also by the Leucyl-tRNA synthetase; the transformed mutant is also able to retain its mitochondrial DNA.
Another synthetase was used to verify *in vivo* the possibility of suppressing the growth defect of the LysG38A biolistic mutant. The Lysyl-tRNA synthetase (LysRS) is a class II synthetase; this class is distinguished by an N-terminal oligonucleotide binding that recognizes anticodon with uridine at the second position. LysG38A mutant, equivalent to the human G8328A, was suppressed by the over expression of LysRS, suggesting that this position could have, also in yeast, an important role in the interaction of the tRNA with the cognate synthetase. Recent *in vitro* experiments demonstrated that G8328A mutation in the human mitochondrial tRNALys leads to a drastic decrease of lysylation efficiency (Sissler *et al.* 2004). In fact Sissler described that the guanine in this position is an essential element for a correct aminoacylation of the molecule.
The specific aminoacyl-tRNA synthetases are not the only proteins which are known to rescue mitochondrial tRNA mutant defects. As previously shown, for example, in tRNALeu mutants the over expression of TUF1 gene, encoding for EF-Tu, was able to rescue the growth defective phenotype on glycerol and to restore a wild-type respiration (Feuermann *et al.* 2003).
LysG38A mutant, characterized in this work, and Ts9 mutant, characterized by Rohou, were suppressed by the TUF1 gene cloned into different multi-copy plasmids. This factor may have a positive effect interacting with the mutated molecules and rearranging the alterated tRNA structures. This hypothesis could be applied also to the synthetases. The studies on the interactions of the tRNA molecules are still in progress; recently, Dale and colleagues suggested a principle of "Thermodynamic compensation" which explains how these complexes could be stable. This principle is based on the combination of strongly binding amino acids with weakly binding tRNA sequences and viceversa (Dale *et al.* 2004).
Moreover, it has been suggested that some mammalian synthetases, as LysRS, are linked to the EF-1 (EF-Tu in yeast) complex for efficient channelling of aminoacyl tRNAs to ribosome (Sang Lee *et al.* 2002). This supramolecular network could play a role as an additional control of the translation.
The aim of this work is to better understand the pathologies related to mutations in tRNA genes and to apply the results obtained in yeast to humans; the high conservation of synthetases as well as EF-Tu, suggests the possibility to candidate these proteins to further studies on human mutated tRNAs. In fact as EF-Tu shows 46 % of identity between humans and yeast, it could be a good candidate for gene therapy experiments.

4.3 Nuclear Background analyses and suppression by EF-Tu

In humans it has been observed that some mitochondrial disorders are characterized by an extremely different penetrance of the disease not only in different individuals but also in different tissues and stages of development (Sasarman *et al.* 2002).
To investigate the possibility that nuclear background may cause differences in the phenotypes of the yeast tRNA mutants, the nuclear context of each mutant described in this thesis was changed. To obtain the same mutated mitochondrial genome in a new context, a Kar1-1, rho° wild-type strain was crossed with a mitochondrial mutant strain (syn- mutants). The results obtained after the crosses revealed that the glycerol growth was more defective when the mitochondrial mutations were associated with a D273-10B/A1 nuclear context compared to the association with an FF1210-6C or an MCC123

context. These results suggest the presence of nuclear elements compensating for mitochondrial defects.
To analyse the possibility that the nuclear background may cause differences in the suppression capability of the EF-Tu, the three different plasmids containing TUF1 gene were used to transform the mutated strains in all three different nuclear backgrounds. Surprisingly, it was found that Ts9 and x14.25 mutants, involving the same bond in different tRNA molecules, respectively in tRNAGly and Thr1, were not suppressed by this factor in the D273-10B/A1 context while they were suppressed when the nuclear context was MCC123. These two mutants showed the same molecular defect in the Northern Blot experiments and therefore it is possible to hypothesize that some nuclear elements may interfere somehow in regulating the mitochondrial transcription levels. Another possibility is that nuclear elements may contribute to the stability of the tRNA structure by preventing its degradation and allowing its participation to the protein synthesis.
The absolute quantification of TUF1 transcript inside the wild-type strains and their isogenic mitochondrial rho° mutants is one attempt to understand its implication in the tRNA biogenesis. In this analysis the rho° cells were considered as a model for the syn- mutants. The RT-PCR makes it possible to compare relative differences between amounts of starting material, whether they be DNA or RNA. The reported results showed an increasing gradient of TUF1 transcript (rho$^+$ FF1210-6C, rho$^+$ D273-10B/A1, rho° D273-10B/A1, rho$^+$ MCC123, rho° FF1210-6C) derived from the RT-PCR analysis. From these data it is possible to draw the following considerations: different nuclear contexts have different levels of TUF1 transcript; the rho° have more TUF1 transcripts compared to their isogenic rho$^+$; it is possible to hypothesize that in the FF1210-6C exists a nuclear element able to compensate for the decreased energetic level caused by dysfunctional mitochondria, in fact, the FF1210-6C wild-type strain has a significant difference in the level of TUF1 transcripts between its rho$^+$ and rho° cells.
It has been observed that low level of cellular energy, due to mitochondrial mutations in tRNA genes, lead to drastic phenotypes when associated with homozygous for particular alleles (for example involved in the intracellular regulation of Ca^{2+})(Johnson *et al.* 2001). Moreover, dysfunctional mitochondria cause in yeast an increase in the expression of some nuclear genes, a mechanism called retrograde regulation. An example of this pathway was observed in respiratory deficient cells in which the absence of TCA cycle activates peroxisomal activity to maintain supplies of biosynthetic intermediates (Epstein 2001).
The preliminary result indicating the difference in the TUF1 transcript level is important, in fact, studies on human pathologies correlated to mitochondrial mutations suggest that the phenotypes depend not only on the specific tRNA mutations but are also regulated by additional genetic and environmental factors. For example, different cybrid cell lines were used by Guan *et al.* to demonstrate that the nuclear genotype determines the penetrance of an A to G transition in the mitochondrial 12S rRNA in an Arab-Israeli family (Guan *et al.* 1996).

4.4 Conclusions and Perspectives

The results described here contribute to widen the current understanding of the yeast system and might provide the basis to derive more general rules, possibly applicable to mitochondrial as well as cytoplasmic tRNAs from other species.
The introduction of mutations in the yeast mitochondrial tRNAs allowed the possibility to investigate the pathogenic potential of some human mutations and to search for more genes that can either suppress or modify the defective phenotype.
In the future it will be possible to plan the introduction into yeast of mutations not yet obtained in tRNALys and Ile. Moreover, it will be possible to mutagenize position 30 of tRNALys to have a condition similar to the human tRNA to demonstrate the specificity of the phenotype.
EF-Tu and aminoacyl-tRNA synthetases could be good candidates to study the effects of their expression in human cell lines from different individuals bearing pathogenic mutations, but not exhibiting a clear pathology.

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